

# Competitiveness across the Atlantic

**Governments disappointed with their economic success turn to the slogan of enhanced competitiveness. But both the British and US governments overlook the simplicities in their case.**

NATIONAL preoccupations with competitiveness are evidently infectious, but not virulently so. In the late 1960s, as the Italian 'economic miracle' of the previous decade faded into mirage, the government of Italy urged on Europe as a whole its diagnosis of 'the technology gap' in which it saw great danger, and which was taken to be a measure of Europe's backwardness compared with the United States. Laughably, in retrospect, Japan was hardly mentioned. Ironically, the cry of competitiveness was next taken up in the late 1970s by President Jimmy Carter's United States, when the worry was Japan, and served as a desultory theme song for the two Bush presidencies. France, a little later, took a still more dramatic line: Jean-Pierre Chevènement, appointed minister of research and industry in 1980, advocated technological change as an instrument of national prowess; thanks to the energy of his more patient successors, the goal has partly become reality. And now the slogan of international competitiveness has spread to Britain, but in a characteristically muted way.

On behalf of their voters, governments seek prosperity in an increasingly global market in which the prizes go to those who can deliver reliable products at low prices or who can make radically innovative products. The slogan of competitiveness has historically been a mark of governments' disappointment at declining international performance. But, curiously, neither in Britain nor in the United States have governments grasped the lessons that need to be learned.

The British case is the more stark. Last year, Britain came sixteenth, by order of gross domestic product (GDP) per head in the ranking of what were then the 24 members of the Organization for Economic Cooperation and Development (OECD). Last year, by the same yardstick, it was seventh out of the 12 members of the European Union (EU). This is a far cry from the 1950s, when, even so, the then government though it worthwhile to mount a detailed comparison of the shortcomings of the British economy through the work of the Anglo-American Productivity Council. The British ministers who last week launched the government's paper *Competitiveness: Helping Business to Win* could do worse than hunt in the archives for those revealing documents.

Meanwhile, the same ministers should read their own document again, asking themselves what impact it is likely to have. The paper acknowledges that Britain's economic performance has indeed declined over the past century, but it then draws a line in 1979 (when the Conservatives came to power) and seeks to demonstrate that things have been improving ever since. This has indeed been a period of

hyperactive government in Britain. Nationalized industries have been privatized, arrangements for higher education and technical training have been in a perpetual state of flux, the rigours of market forces have driven a kind of darwinian evolution of British industry into occasional patchy extinction, and now the public support of research is going through root and branch upheaval. To be fair, many of these changes may contribute to enhanced competitiveness, even if some of them are gambles.

But the British problem is deeper than that, and in two important ways. First, economic growth seems inseparable from price inflation. And even now, when industrial production is rising towards the level reached in 1991, there is talk of increasing interest rates again to keep inflation in check. The other peculiarly British difficulty is that public policy lacks consistency of the kind conspicuous in France. Historically, the electoral process has become a means by which public policy is moved through right angles, disconcerting trading partners and the rest of the world. In that sense, British competitiveness (or the lack thereof) may stem from its perverse boast that it lacks a written constitution. The government's paper promises further editions on the same theme. It should take up both these questions the next time round.

The US difficulty is different. There are the same long-standing difficulties about the quality of public education and the means by which a government can reliably and legally stimulate innovation, but the US economy as such remains in many ways the most flexible in the world. The drag is that the size of the federal deficit has depressed the dollar, and that the continuing trade deficit depresses it further. So the administration has become itself into a kind of export salesman, as typified in the past few weeks by its arguments with the governments of China and Japan.

Predictably, President Bill Clinton has been forced by reality to retreat from the hard line that a continued low-tariff relationship with China would be possible only if China quickly became more like a Western democracy; he should not have put himself in such a brittle position in the first place. But the administration has relatively bigger sticks with which to thump a more compliant Japanese government. By October, when a decision must be made, it will seem very odd that China will have been accorded 'most-favoured nation' status on trade, while Japan may be among the least favoured trading partners of the United States. "So what?", the administration in its present mood will ask. Let us hope that it does not invite an unwelcome answer. □



# The newest US justice

**Judge Stephen Breyer would bring to the US Supreme Court special expertise in science.**

IN nominating Stephen G. Breyer of the federal district court in Boston to the US Supreme Court, President Bill Clinton has made an excellent choice, but for the wrong reasons. Breyer, who is 55, has degrees from Stanford and Oxford as well as a law degree from Harvard, where he is a professor. He is widely admired by colleagues, who describe him as intellectually astute, widely read in law and literature and utterly fair. Although he has been widely described as a conservative jurist, his greatest strength is his willingness to judge cases on their merits; all too often, Supreme Court judges are encumbered by the expectation that their political philosophies (right or left) and even prejudices will take precedence over facts and the law.

Breyer's appointment is especially welcome because it will bring rational jurisprudence to bear on the complex issues in science and technology likely to find their way to the Supreme Court in the coming decade. Breyer knows about regulatory issues in novel industries, from aerospace to biotechnology. His quality is illustrated by his decision, during a sabbatical from Harvard, to immerse himself in a study of risk analysis to produce a book (*Breaking the Vicious Circle: Toward Effective Risk Regulation*, Harvard University Press) that cannot now be had in Washington for love or money.

His good sense is illustrated by the important case involving the US Superfund law for cleaning up toxic waste, when he argued that public misperceptions of risk can distort reality and skew government policy. Thus the law demands that the last 10 per cent of chemical waste at a dump site in New Hampshire be cleaned from the soil at a cost of \$9.3 million despite what the judge said was valid scientific evidence to the effect that the soil had so little toxic material that children could safely eat it. Because the site is a swamp, he added, there were no dirt-eating children to protect in any case. But he also carries solid environmental credentials. He has upheld a ban on land-destroying dune buggies on the fragile Cape Cod seashore and ruled against an oil refinery in northern Maine that posed a genuine risk to endangered species.

So how can the reasons for this paragon's nomination to the Supreme Court be called wrong? Mainly because Clinton foolishly let it be known that he would prefer to have named a good friend from Arkansas (who has cancer) or Bruce Babbitt, the strong, sensible, but controversial Secretary of the Interior. Babbitt, a former governor of Arizona, quickly made his mark as a member of Clinton's cabinet with a sensible strategy for dealing with conflicts between development and endangered species by promoting a broad ecological approach to environmental issues. But he also made headlines (and enemies) when he courageously (and correctly) attempted the politically unthinkable by seeking the preservation of western lands by increasing the derisory fees

paid by cattlemen, miners and others for their use, free from the obligation to restore what they destroy.

But Babbitt's nomination was stillborn. When Senator Orrin Hatch (Republican, Utah) threatened to fight it in the Senate, Clinton backed down; although Babbitt would probably have won through, Clinton (with so much else on his hands) chose not to make a fight of the issue. By having said so clearly that he wanted Babbitt and then shying away with hardly a word, the president did nothing to enhance his reputation as a leader of courage or a man of principle. Indeed, while praising Breyer's qualifications, Clinton has also let slip to colleagues that he had not found the new judge a scintillating luncheon companion. Luckily the new Supreme Court justice (his confirmation is all but inevitable), who has endured the companionable jogging ritual, has had the good manners to stay silent on his luncheon conversation. His interests, thankfully, seem to be with the court. □

## Resurgent infections

**British newspapers' alarm about a "killer bug" are a reminder that infections are still with us.**

THE British public health authorities are right to discount the alarming tales being told in British newspapers about the emergence of a new "killer bug", but they have a problem on their hands. Since the beginning of this year, fifteen cases of the condition known as necrotizing fasciitis have been recorded and have been attributed to infection by group A *Streptococcus*. The condition is one in which subcutaneous tissue is destroyed by the infecting organisms, but those affected may die of bacterial shock, disseminated blood coagulation or respiratory or renal failure. Only two of the fifteen known patients have survived. What seems to have alarmed the physicians is the speed with which those infected have been overwhelmed.

The condition of necrotizing fasciitis is by no means unknown, but has become more common in the past decade. Most (but not all) of those infected in the past few months in Britain had been in hospital for surgery, but there is little doubt that the publicity given to a few cases has prompted physicians to be more zealous in reporting similar cases that have come their way (some of which have not been linked with *Streptococcus A*). Bizarre though it may seem, unless antibiotics are given quickly, survival may turn on the physical removal of flesh by surgery.

Even so, none of us should be all that surprised by this development. For several years, there has been mounting concern that intractable infections of various kinds could prove a resurgent threat to public health, believed in the 1960s to have been routed by antibiotics (and synthetic pesticides). AIDS is the most worrying novelty of this kind. Antibiotic-resistant bacteria are another. In all cases, the urgent need is that the molecular mechanisms of their persistence and pathology should be understood. That is where the British authorities must put their energy. □



# Clinton administration urges basic research boost in new budget plans

**Washington.** The Clinton administration has told all agencies and government departments with a responsibility for research to boost spending on basic science in their budget requests for the 1996 financial year, which starts on 1 October 1995.

The White House instruction is contained in an unpublished memorandum from Leon Panetta, director of the Office of Management and Budget (OMB), and Jack Gibbons, director of the Office of Science and Technology Policy (OSTP), to all agency and department heads.

It confounds early expectations that the administration would favour technology over fundamental science in allocating its annual \$73-billion research and development (R&D) budget. The memorandum also tells agencies and departments to increase the amount of R&D money going to the universities, and to subject more of their spending to thorough peer review.

Panetta and Gibbons do not specify where the extra money will come from. But the growth is likely to be at the expense of intramural government R&D. The largest such programmes are those at the Department of Energy, the Department of Defense (DoD) and the National Aeronautics and

Space Administration (NASA).

The 18-page memorandum outlines the "R&D broad policy principles, goals, priorities and evaluation criteria" for all agencies as they prepare budget submissions for the 1996 fiscal year. It is based on work carried out by the nine subpanels of the National Science and Technology Council (NSTC) which President Clinton established last November (see *Nature* 366, 393; 1993). Its suggestions on basic research appear to reflect the impact of the fundamental science and engineering subpanel, which has acted quickly to build a case for basic science.

"Agencies are strongly encouraged to measurably increase their absolute investment in (1) fundamental (basic) research and (2) research supported in academic institutions in the FY96 budget submissions," Gibbons and Panetta say in the memorandum. "In addition, merit reviewed with peer evaluation research, especially that research conducted at academic institutions, is of highest priority. Thus, research not subjected to peer review is expected to decline."

They identify six target areas "needing new or additional emphasis in FY 96". These are: a healthy, educated citizenry; job creation and economic growth; [US] world lead-

ership in science, maths and engineering; improved environmental quality; harnessing information technology; and enhanced national security.

In each case, fairly detailed priorities are listed for extra government investment. On health, for example, the memorandum singles out vaccine development for AIDS and other diseases, methods for preventing HIV transmission and a range of nutritional, environmental and social approaches to the improvement of public health.

Genetics, structural biology and neuroscience are all emphasized. But the health section has a 'back-to-basics' tone, with less emphasis on high-tech medicine and more on meeting the health needs of America's poor.

On the issue of economic growth, Panetta and Gibbons ask for further expansion of a number of industrial programmes already initiated, including the Advanced Technology Program. But a notable omission from the goals for aeronautics are the plans of Daniel Goldin, head of NASA, for R&D on supersonic civil aircraft.

As well as promising to expand basic research funding, Panetta and Gibbons pledge to start "an interagency program of competitive awards" to fund new university buildings and equipment in 1996.

Among many environmental issues raised, there are specific pledges to launch large-scale pilot studies in 1996 to test integrated ecosystem management, and to initiate "demonstration projects to determine the feasibility of biomass as a major energy supply technology". Predictably enough, the information technology section emphasizes applications and means of access for the information superhighway.

The section on national security is short on specifics, beyond the need for better intelligence gathering and work "to make land mines self-neutralizing after a compressed period of time". This suggests either that the DoD has plenty of secret projects in hand, or that it is having trouble justifying its \$40-billion share of the annual research and development budget.

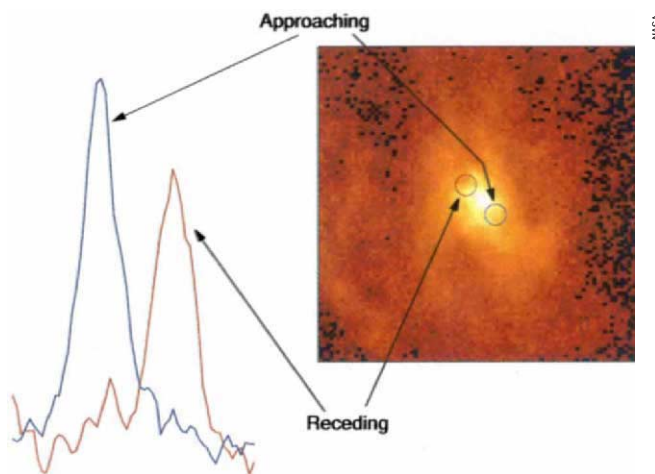
Alternatively, the DoD may think it has better things to do than explaining itself to the NSTC — a body that has yet to prove its authority. The congressional warlords who traditionally carve up the science budget were always expected to resist any encroachment by Gibbons and the NSTC on their turf (see *Nature* 365, 195; 1993). But Panetta is a powerful figure in Washington, and this assertive and purposeful memorandum is a clear sign that the NSTC means business.

Colin MacIlwain

## Hubble reveals 'proof' of black hole

**Washington.** Almost everything in astronomy is uncertain to within a factor of 2. But even allowing for that uncertainty, there was general acceptance among astronomers last week that Holland Ford and his collaborators at the Space Science Institute in Baltimore, Maryland, have demonstrated the existence of a giant black hole at the centre of the galaxy M87.

By using the Hubble Space Telescope to measure the Doppler shift of emission lines from doubly ionized oxygen about 20 parsecs (65 light years) away from the centre (see above), the astronomers have determined a rotation velocity of about 550 km per second for the gas, and estimated a mass interior to that point of  $2-3 \times 10^9$  solar masses.



The mass in this small region is so great that it is very hard to conceive of any explanation other than a giant black hole. If the mass were contained in stars like the Sun, they would be packed 75,000 times more densely than in the solar neighbourhood. But this explanation is very unlikely, as there is not enough light coming from this region to allow the presence of so many stars.

Leslie Sage



# US government rejects plea for nuclear waste inquiry

**Washington.** The US Department of Energy (DoE) has acknowledged that it will not be able to meet its obligation to accept nuclear waste from power stations in 1998, and has invited the operators of such stations to discuss alternatives — such as compensating them for storing waste locally instead.

The department's action, announced last week in the *Federal Register*, has been seized on by its critics as evidence that the government's nuclear waste policy is in disarray. But Hazel O'Leary, the Secretary of Energy, promptly rejected a call from a broad coalition of environmental groups for a presidential commission to find new approaches to the management of nuclear waste.

Such a commission was proposed two weeks ago in a letter to President Bill Clinton from the Sierra Club, Public Citizen and dozens of other environmental groups, and endorsed by 40 senators and congressmen. But O'Leary says that a commission would take 15 months to establish. "I just don't have the time," she adds.

The DoE's notice states its view — which is likely to be challenged in the courts by electricity utilities — that it has no legal obligation to take the waste, because it has no repository. The notice raises the question of whether an interim central store is needed, and of whether the department should release compensation from the \$6 billion nuclear waste fund to help utilities to pay for temporary local storage.

Utilities and other interested parties have four months to respond to the notice. O'Leary is thought to favour subsidizing local storage, leaving the utilities to fight for and build an interim central store if they so choose, and proceeding with the painstaking process of constructing a permanent store at Yucca Mountain, Nevada, for completion by 2010 at the earliest.

A DoE "stakeholders' meeting" on high-level nuclear waste held in Las Vegas last week was told that the use of standard, multi-purpose canisters would assist the safe storage of waste on local sites until central storage became available at some future date.

But environmentalists say that a commission is needed to establish a national consensus that would lift the issue from its current quagmire of doubt. "We need an independent review because no-one really knows what we're doing," says Martin

Gelfand, research director of a Washington-based lobby group, the Safe Energy Communication Council. He professes surprise at DoE's dismissal of the proposal for a commission. "We don't understand why they are so headstrong, because they don't know where they're heading," he says.

Some environmentalists would certainly like the government to procrastinate on nuclear waste management, hoping that the resulting logjam would cripple the nuclear power industry. But they deny that the call for the commission is a stalling tactic.

They say that the long-delayed plans for permanent storage of 70,000 tonnes of high-level civil waste at Yucca Mountain and military waste at the Waste Isolation Pilot Plant near Carlsbad, New Mexico, will never be realized and should be replaced with a programme determined by a presidential commission via a "public, scientifically-sound process".

But with no central storage facility in sight, nuclear power stations face mounting difficulties. "Within four years, 23 units will run out of storage space, and by the year 2010, 73 units will run out," says Phillip Bayne, president of the Nuclear Industry Institute, the main industry lobby group.

In contrast with the emerging pattern in Europe, central storage is the industry's favoured option, probably because local politics in the United States makes local storage highly problematical.

Plans for a Monitored Retrievable Storage facility for interim storage have been pursued by an independent agency, the Office of the Nuclear Waste Negotiator. But negotiations with several Indian tribes to take waste on reservation land proved politically explosive, and Congress has cut off its money from the end of the year.

The first and most organized tribe — the Mescalero Apaches in New Mexico — is now pursuing the idea of a privately built interim store. This week, 33 utilities are expected to agree to proceed with the detailed planning phase of such a store, which would hold 20,000 tonnes of high-level waste and cost a moderate \$150 million to build. They would then apply for a licence from the Nuclear Regulatory Commission.

This plan will meet fierce opposition from environmentalists, and from politicians such as Senator Jeff Bingaman (Democrat, New Mexico), whose amendment to an appropriations bill last autumn killed off the negotiator's office. If the Mescalero private store dies too, the United States is likely to see little real progress on the high-level waste issue apart from more local storage, and endless scientific reassessment of the Yucca Mountain site. **Colin Macilwain**



**O'Leary: claims a lack of time.**

# UK nuclear company funds broad study of genetic data

**Manchester.** A plan to collect and analyse samples of genetic material from nearly every baby born in west Cumbria in the north of England over the next five years has been approved in principle by an ethics committee of the region's health authority.

John Burn and Louise Parker of the University of Newcastle upon Tyne have been guaranteed about £300,000 (US\$450,000), to cover at least the first three years of the project, by the Westlakes Research Institute, an independent organization set up outside Whitehaven, Cumbria, by British Nuclear Fuels (BNFL).

BNFL has struggled for a decade to shake off suspicion that its reprocessing plant at Sellafield in Cumbria might be responsible for a cluster of childhood leukaemia cases in the nearby town of Seascale. Last year, a High Court judge dismissed allegations that men exposed to radiation at the plant were more likely to father leukaemic children.

The company now hopes that it can allay lingering doubts with this new research and "prove there is no difference between the genetic make-up of children born to Sellafield fathers and those from the rest of the region", says a company spokesman.

Intended to involve 10,000 children, the project is likely to be the biggest single study so far in population genetics. It must now go through an extensive public consultation process to establish strict guidelines for handling an unprecedented mass of confidential genetic information. Barring delays, consenting parents could be providing samples of afterbirths early next year.

Burn says that BNFL's generous funding, which is far higher than its statutory requirements to monitor employees and the local environment for radiation exposure, is intended as both an effort to legitimize research sponsored by the nuclear industry and a lure to attract more scientists to the relatively isolated region.

To win preliminary assent from the West Cumbria Research Ethics Committee, Burn and Parker had to incorporate into their proposal a coding system to ensure the anonymity of donors. Before giving written and oral consent, prospective parents will see a video explaining how genetic information from their baby will be used.

Burn insists that he will consider using the code only when doing so will be of undisputed benefit to the child's health. He intends to put together an ethics committee, of nationally prominent geneticists and local people to decide what situations might merit such action. Its recommendations would have to be approved by the West Cumbrian committee, which Burn describes as "no pushover". **James Younger**

# Ministries clash over rival visions of the future

**Tokyo.** The appearance of rival reports published within days of each other in Tokyo by two separate government departments — offering very different visions of Japan's future information infrastructure — represents an early skirmish in what promises to be a lengthy struggle.

The reports were drawn up by the Ministry of International Trade and Industry (MITI) and the Telecommunications Council, a high-level committee that advises the Japanese Ministry of Posts and Telecommunications (MPT).

Historically, MITI has overseen Japan's computer industry, while MPT has controlled communications and broadcasting. But the spread of digital technology means that telephones and televisions increasingly resemble computers, and this fact has precipitated an interministerial turf war.

One big difference between the reports is that while MITI's *Advanced Informationization* [sic] *Program* focuses on the importance of promoting the use of the network,



MPT's *Establishing a New Information Infrastructure for the 21st Century* emphasizes construction of the network itself.

The MPT report envisages a three-stage project to lay optic fibres to all Japanese homes and offices by the year 2010.

The cost would be between ¥33,000 and ¥53,000 billion (\$317 to \$510 billion). The report makes no attempt to indicate where such huge sums would come from. Nippon Telegraph and Telephone (NTT), Japan's semi-privatized telecommunications giant, has offered to do the job, but only if it is allowed to raise its tariffs, and MPT has recently ruled out such an increase.

The report drew immediate criticism for its insistence that Japan's information infrastructure should be made up exclusively of optic fibre, rather than a mixture of connection schemes including satellite, wireless and copper cable.

Less controversial is its suggestion that, for the sake of efficiency, the network should be built by the private sector, with the government's role limited to providing tax incentives and low-interest loans.

This apparent hands-off policy represents a U-turn from MPT's recommendation last autumn that a public corporation be set up under the ministry's control to do the construction. Since making that proposal, MPT has come under fire — most notably from its former vassal, NTT — for the repeated failures of its *dirigiste* policies.

Many Japanese now realize that red tape, for which MPT is responsible, is the main reason that their country's cable television and mobile communications industries lag so far behind those in the West.

But some observers wonder whether this enthusiasm of telecommunications officials for private-sector initiatives is as wholehearted as it seems. "They know they have to do some deregulation," says Izumi Aizu of the Center for Global Communications, a small but influential private think-tank. "But if you read the report carefully, it's clear that they want to remain the major player."

Giving the private sector its head through deregulation and liberalization also figures as one of two main themes outlined in MITI's report. The other, according to one MITI official, is that "it is important for government to act as a very advanced user of information technology".

The report's concrete proposals include

a recommendation that 100 schools be connected to the Internet to demonstrate the educational benefits of computer networking. In contrast, the MPT report makes no mention of networking.

Emphasizing the use of the network is a new twist for MITI, whose traditional role has been to protect and promote the growth of Japan's domestic computer industry. Shumpei Kumon, a leading Japanese networking advocate, says that MITI seems to have realized that "what is important at this moment is not to protect and promote industry, but to promote the development of networking, using the most advanced technology that can be imported".

But although the bureaucrats have belatedly realized that building an information infrastructure is important for Japan's future, Kumon complains that they still "have no clear vision of what the information revolution means".

Involving schools with networking means that MITI must win the support of the Ministry of Science, Education and Culture for its proposals. The trade ministry is trying to set up an inter-agency task force under the leadership of the prime minister's office to push for further deregulation of telecommunications and the development of networking. Other agencies, including the ministries of education and health and welfare, are said to be enthusiastic about participating in the task force's activities. Only MPT appears reluctant to join.

**Bob Johnstone**

## Wool slump leads to research cuts

**Sydney.** Changing national priorities, budget cutbacks and a slump in the Australian wool industry have led to the loss of 138 jobs and 10 research sites by Australia's main agricultural research organization. The cuts were announced by the agricultural research section of Australia's Commonwealth Scientific and Industrial Research Organisation (CSIRO) in a move designed to reduce its A\$126 million budget by 13 per cent.

Proposals circulated last week to staff of the CSIRO Institute of Animal Production and Processing, which accounts for 15 per cent of the organization's overall budget, would cut overheads by closing down 10 research sites and consolidating operations on the remaining 17. The institute intends to eliminate 174 positions, including 21 researchers, out of a staff of 1,554. But some staff and researchers will be re-deployed, and new staff may also be hired, so that the net loss is likely to be 138 staff — including only three researchers.

Alan Donald, the director of the institute, says the plans have yet to be discussed with staff, and that any proposed budget cuts

need the approval of the CSIRO board.

CSIRO itself has had its budget cut this year by A\$20.5 million (see *Nature* 369, 90; 1994), and an internal review of research priorities has led to a decision to shift funds from agriculture into areas such as manufacturing and minerals.

But this will account for a reduction of only A\$5.5 million in the institute's budget. According to Donald, funds from industry are also expected to fall by A\$11.5 million to A\$22.5 million, mainly due to a slump in the Australian wool industry.

The shake-up also reflects a shift in CSIRO's priorities within agriculture away from 'production' issues, such as animal health, into 'processing' research — for example, developing ways of making wool more competitive with natural fibres.

The rationalization of sites is expected to include combining the Division of Animal Health's laboratory, at present adjacent to Melbourne University, with the Australian Animal Health Laboratory at Geelong, a regional centre southwest of Melbourne.

**Mark Lawson**



# UK gene-release suspended after protests

**London.** Researchers planning to release a genetically modified virus in a field in Oxfordshire, England, have suspended their experiment for 14 days following protests from scientists, local residents and environmental and conservation groups.

The research was due to begin last week. It involves spraying caterpillar-infested cabbages with an insecticidal baculovirus (*Autographa californica* NPV) carrying a scorpion toxin gene, and is being led by David Bishop of the Natural Environment Research Council (NERC)'s Institute of Virology and Environmental Microbiology in Oxford. Bishop maintains that the risks and environmental impact are "minimal or nil". He points out that the work is a repeat of a similar experiment at the same site last year, and that it has already been given full safety clearance by the Department of the Environment (DoE).

But opponents disagree with Bishop that the safety of the experiment has been adequately examined. They suggest, for example, that potential dangers arising from the organisms's genetic stability and host range (see *Nature* 353, 394; 1991), have been underestimated. They are also angry that their objections to the release seem to have been ignored by the department, and point out that they were not even given enough time to register their objections.

George Smith, a materials scientist at the University of Oxford who lives near the release site, has called for a "full, in-depth, wide-ranging public inquiry" into procedures for granting consent to releases of genetically modified organisms. He argues that current arrangements for public consul-

tation "are inadequate to deal with matters of such widespread concern and importance".

But the DoE points out that neither it, nor its Advisory Committee on Release to the Environment (ACRE), has a statutory obligation to consult the public about such releases. The only obligation on researchers is that they place an advertisement of their proposed work in a local newspaper.

The NERC team did this. It also invited the public to seek additional information from — and to send comments to — the DoE. But the trial was given the go-ahead before the department sent Smith the documents he required to carry out his own review of the proposal.

Smith, whose protests are being backed by Friends of the Earth, complains that he was given no indication of the decision-making time-scale, and was never informed by the department that the test had been

approved, even though he had submitted a formal objection a day after the advertisement appeared.

Earlier last week, lawyers acting for the protesters lodged a complaint with John Gummer, the Environment Secretary, claiming that the department had created a "legitimate expectation that the objections of members of the public would be taken into account" and had failed to fulfil this commitment. They also draw attention to discrepancies between the advertisement and the public register about the sites's exact location.

But a spokesman for the department says that it has "complied fully" with legislation. Although a meeting was held on 24 May at which the department discussed the complaints with its advisers, officials insist that this was a routine event, and was not prompted by the Oxford situation.

**Peter Tallack**

## Nature, not levees, blamed for flood

**Washington.** The Great Mississippi Flood of 1993 was a rare natural event whose impact was not significantly worsened by existing river management policies, according to a White House task force that had been charged with framing a policy response to the flood.

General Gerald Galloway, the chairman of the task force and a US Army engineer, told a Senate hearing last week that neither the loss of natural wetlands to farmland in the Mississippi flood plain nor the use of man-made levees to constrain the river banks had contributed significantly to the severity of the flood.

Nonetheless, a draft version of the task force's report says that the flood should "serve as a catalyst" for change, and proposes action to encourage a better balance between levees and other, non-structural approaches to the management of flood plains.

The report recommends new legislation to establish a "national paradigm for floodplain management", coordinating federal, state and local action, and giving clear responsibility for leadership to state governments.

It also advocates the re-establishment of interstate river basin commissions on the Upper Mississippi, the Missouri and elsewhere, and the use of the Upper Mississippi as a cross-agency ecosystem management demonstration project.

Members of the Senate Environment and Public Works Committee, chaired by Max Baucus (Montana, Democrat), said that they welcomed the report. The committee is likely to support floodplain legislation which, Baucus hopes, will revise the mandate of the

US Army Corps of Engineers.

American Rivers, an environmental group opposed to levees, says the report shows that "this 200-year-old experiment by the Corps of Engineers has failed". But the draft report says that levees prevented \$19 billion of damage on top of the estimated \$12–\$16 billion flood damage.

John Zirschky, acting assistant army secretary in charge of the Corps, says that the Corps will welcome a change in mandate

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UNAVAILABLE  
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REASONS

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**Last year's flood: "a catalyst".**

enabling it to take into account non-economic factors (such as environmental conservation) in its work. Galloway says the Corps "needs funding to use some of the techniques it has known about for years".

There remains a sharp disparity of view on how quickly levee repair work is actually proceeding on the Mississippi (see *Nature* 364, 747; 1993). Senator Chris Bond (Republican, Missouri) says that "unconscionable" bureaucratic delays meant that "only 19 out of 500 levees were fixed by December". But Zirschky says that repairs to 71 per cent of damaged levees are complete or under way.

**Colin Macilwain**

## French agency opens laboratory in Japan

**Paris.** France's Centre National de la Recherche Scientifique (CNRS) has opened its first laboratory in Japan. CNRS says that the Laboratory for Integrated Micromechatronic Systems (LIMMS), to be based at the University of Tokyo, will help to balance the current trend where more Japanese researchers work in France than the other way round. By associating the LIMMS with three existing laboratories in France, CNRS also hopes to improve the reintegration of French researchers returning from Japan.

LIMMS will not have its own buildings, except for an office for the director, and the researchers will instead be spread out in Japanese laboratories to "avoid risk of isolation". The laboratory is seeking partners from French public and industrial sectors. CNRS opened a permanent office in Tokyo in 1992. It also offers courses in Japanese to its researchers.

**D. B.**



## ANC-led government 'will protect funds for basic research'

**Cape Town.** In her first scheduled engagement as Deputy Minister of Arts, Culture, Science and Technology, Mrs Winnie Mandela last week emphasized that science and technology have an important role to play in meeting the priorities of the new government.

Mandela's speech was due to be delivered to a regular meeting of the Science and Technology Initiative (a grouping of representatives from the science councils, universities, state-owned corporations and the private sector) at Midrand, halfway between Pretoria and Johannesburg. But having turned up two hours late — and after the meeting had officially closed — she ended up addressing those of the committee members who were still present.

The speech reiterated that the coalition government, led by the African National Congress (ANC), is likely to follow a path "which will direct research and development activities towards economic growth, competitive advantage and meeting the needs of all our people". But Mandela assured the research community that the ANC still held a positive attitude towards basic research. "We do not accept the view that basic and applied research should be in conflict with each other," she said.

In her speech, Mandela also referred to the legacy of scientific illiteracy and the shortage of technical expertise in the country arising from apartheid, pointing out that 96 per cent of the country's engineers, and 89 per cent of its scientists, are white.

She praised the Science and Technology Initiative for commissioning a report on affirmative action programmes for science councils, which was tabled at the meeting.

No appointments have yet been made to any positions in the new ministry. But last Friday Mandela announced the members of an *ad hoc* committee set up to advise the minister, Ben Ngubane, and herself.

This includes Koos Pauw, deputy director-general at the Department of National Education; Roger Jardine, ANC science and technology policy coordinator; Michael Kahn, from the Centre for Education Policy Development; and Isaac Amuaah, from the Foundation for Research Development.

The committee will be responsible for targeting science and technology policy towards the goals of the ANC's reconstruction and development programme. There are few indications as to what exactly this will involve, except that Mrs Mandela's speech made reference to the importance of environmentally clean technology. It is also considering introducing incentives to persuade industry to spend more of its profits on research and development. **Michael Cherry**

## NASA head urges new focus on search for habitable planet

**Baltimore.** Daniel Goldin, the administrator of the National Aeronautics and Space Administration (NASA), suggested last week that the agency should adopt as a new 'unifying mission' the search for a habitable planet close to a nearby star.

Addressing the spring meeting of the American Geophysical Union (AGU), Goldin said that such a goal would allow the agency to continue to pursue much of the science that it is currently supporting. But it would also give NASA a concrete objective that the American public would understand.

Before the collapse of the Soviet Union, said Goldin, the space agency had been given a simple task that the whole of America endorsed: beating the "evil empire". But with the ending of the Cold War, that task had become meaningless.

The public want to know about science, he said, but finds little of interest in NASA's current space programmes. The key is to find a unifying theme, and the search for a habitable planet could fit the bill. "We need goals to integrate the space sciences and give the public something to understand."

The search for such a planet, which might be within ten or so light years from Earth, would require missions such as those already planned to Pluto, Mars, and the asteroid belt, in order to understand how planetary systems are formed. It would also be necessary to understand star formation, as well as phenomena such as protoplanetary disks.

"This is the 75th anniversary of the AGU,"

Goldin said, stressing that his suggestion was intended primarily as a way of opening up a debate in the scientific community. "It would be fantastic if, at the 100th anniversary, someone could present an image of a planet which is habitable, and which is not the Earth."

The NASA administrator claimed that even ancient civilizations had recognized the need for a vision. Rome had eaten itself to death when it stopped building roads and monuments and, if it continued to focus on short-term problems such as fixing the budget deficit, the United States was in danger of doing the same.

Challenged after his talk on why, if he felt NASA ought to pursue such a vision, its earlier Search for Extraterrestrial Intelligence (SETI) programme has been cancelled, Goldin pointed out that this decision was taken by Congress, not the space agency.

He also warned that, even if Congress were to cancel the international space station, the "feeding frenzy" that is likely to follow would not necessarily mean that the money is reallocated to alternative space projects.

Goldin's call for smaller, cheaper missions with a unifying focus has widespread support both inside and outside the agency. But many are sceptical about whether, even if the search for a habitable planet is a goal that fires the public's imagination, Congress — given its attitude towards SETI — would find it acceptable. **Gabrielle Walker**

## Switzerland eyes bill for Framework

**Basel.** Swiss scientists' fears that government funds for basic research could be sacrificed in order to support Switzerland's participation in the European Union's fourth five-year Framework programme seem to be justified (see last week's *Nature* page 264).

Last week, the government put forward a bill, due to be discussed by parliament later this month, asking for an additional SFr261 million (US\$180 million) to make up an expected shortfall between 1995 and 1999. The shortfall has been created by a difference between Switzerland's original estimates of the costs of its participation in Framework and subsequent calculations based on the increased costs of the new programme.

But the government also suggests that SFr176 million of this should be transferred from other budgets. SFr133 million would be taken from the budget

allocated to the three priority programmes of the Swiss National Funds, covering environment, biotech and informatics. The rest of the savings will be shared among the research budgets of government agencies dealing with the environment, agriculture and economics.

Two weeks ago, a letter from five Swiss Nobel prizewinners expressing concern that Swiss research may be asked to pay too high a price for participation in the EC framework programme was leaked to the press. According to newspaper reports, the scientists argued that national research is more valuable, and of higher quality, than that carried out within the Framework programmes.

But Richard Ernst from the ETH Zurich, Switzerland's federal university, has condemned the leak. He says that the letter had been misinterpreted.

Oliver Klaffke

# Greeks seek to balance funding from Brussels

**Athens.** The European Commission (EC) has taken over from the national government as the largest single sponsor of Greek research. In 1986, five years after Greece joined the European Union (EU), money from abroad was equal to only three per cent of government spending on research and development; by 1991, almost a third of research funds came from the EC alone.

The Greek government, which has deliberately set out to use the EC as a major source of research funding, is unembarrassed by this trend. "Brussels has helped us create our critical mass of scientists and improve the quality of our research," says the secretary-general for research and technology, Nicos Christodoulakis.

But some scientists fear that the result could be damaging for both Greek science and the country. They are urging the government to channel more domestic funding into research if it wants to secure its future.

Greece spends less on research — 0.45 per cent of its gross national product — than any other country in the EU. But it receives one of the highest levels of research funding from the EC in relation to its size.

In addition to 'structural' funds, which are distributed to governments of poorer EU countries to improve their infrastructure and

can be used to create research facilities, Greece won 3.6 per cent of the money distributed under the EC's third Framework programme, covering the period 1989–93. This is three times higher than the proportion of the Greek population within the EU, and six times higher than the proportion of scientists working in Greece.

As a result, many individual research institutions have become heavily dependent on the EC. More than half of the total budget of the Institute of Molecular Biology and Biotechnology (IMBB) in Crete, for example, comes from Brussels.

Researchers at the institute acknowledge that, without such support, the institute itself would probably never have been built. But there is resentment about the extent to which its fortunes depend on an agenda set in Brussels. "We sometimes feel we are simply working on contract for the EC," says director George Thireos.



**Christodoulakis:**  
'build infrastructure'.

## EC backs industrial studentships

**Athens.** Keen to promote tighter links between research and industry, the Greek government has, with backing from the European Commission (EC) in Brussels, just launched a joint PhD programme which will fund 500 studentships with a strong industrial bias.

Greek industry has one of the lowest expenditures on research in Europe, providing only a quarter of the country's total spending on research. The government has been trying to encourage industry to make more use of scientific results. But the effort has been small-scale, and hampered by the low level of industrial activity in Greece's predominantly rural economy, as well as the lack of an indigenous tradition of industrial innovation.

Isolated efforts have been made to improve this situation. The Foundation for Research and Technology Hellas (FORTH), for example, set up seven research institutes in the 1980s with the intention of turning the results of their basic research into marketable products.

This has had some success. In Crete, for example, the Institute for Molecular Biology and Biotechnology (IMBB) manufactures and sells restriction enzymes, oligonucleotides and immuno-diagnostic kits. But only the immuno-diagnostic division has so far been

converted into a spin-off company. "A major problem we face is the low demand for R&D by Greek industry" says FORTH director Eleftherios Economou.

Now, in order to improve the overall situation, the government has drawn up a four-point plan which it hopes will be funded through the structural funds of the European Union. One element is the new PhD programme, which will cost ECU9.5 million over six years.

In addition, plans for four technology parks will be completed by creating liaison offices at each. The general secretariat for research and technology will also help to finance the setting up of a number of private institutions to help Greek companies to acquire foreign technology. And a number of bureaux will be set up to bring together industry and researchers.

The government's determination to make science serve the economy is underlined by its decision to restrict all new research grants to applied research. But this move is being contested by the academic community, worried that the country's basic research base will be severely weakened. "The EC does not give money to investigate brand new research," says George Thireos, director of the IMBB. "We need seed money from the government if we are to have a future."

**Alison Abbott**

Similarly most project money at the two biggest research institutions in Athens — the Demokritos research centre and the National Hellenic Research Foundation (NHRF) — also originates in Brussels. "For Greece, EC funding is not the cherry on the cake, but the cake itself," says Ion Siotis, director of nuclear physics at Demokritos.

Panagiatis Manakos, the director of Demokritos, points out that as a result most of the government's domestic spending on research is used to match EC funds. This, he says, prevents Greece from developing a national science policy of its own.

He also complains that Greece has few plans to sustain investments made by the EC. For example, more than ECU1 million of structural funds were used to build a library at Demokritos. But the centre does not receive any government support for journal subscriptions. Others point out that Greece, like other small EU countries, has a small voice in shaping EU research policy.

But the pleas of the scientific community find little echo in government corridors, where EC support is acknowledged to have played a significant part in building up the country's research infrastructure. Christodoulakis sees little point in raising domestic funding for research until this infrastructure is improved still further.

Of greater concern to the general secretariat is the fact that the domestic provision of matching funds has recently been cut back severely. In 1992, the government provided sufficient funds to match all those received from the EC; but last year it matched only 10 per cent of EC grants.

As a result, recipient research centres have had to find the money from elsewhere, and often admit to having used 'creative accounting' to meet EC regulations. The secretariat is keen to change this situation, and has now asked for a share of the economics ministry's reserve funds to make up the difference.

**Alison Abbott**

## French back EU efforts on research

**Paris.** Officials in the research and environment directorates of the European Commission have been cheered by the results of a survey on attitudes towards the European Union (EU) carried out by the French newspaper *Libération*. This revealed that almost three-quarters of the French population approve of the EU's policies on research.

The survey, whose results were published last week, showed that 53 per cent felt similarly about EU environment policy. In contrast, two-thirds of those questioned felt that EU policies on employment and agriculture are going in the wrong direction. □



# DNA profiling on trial

SIR — D. Balding and P. Donnelly<sup>1</sup> are correct in their point that the match probability arising from a DNA profile is not the same as the probability of innocence of the suspect — the probability of innocence depends upon the totality of the evidence. But their hypothetical calculation to illustrate the difference between the two probabilities is not helpful.

They consider a case in which an individual has a match with a scene-of-crime DNA, which can be calculated to have a probability of one in a million, but this individual has, on the basis of the evidence other than the DNA profile, approximately a one in a half million chance of being guilty. Balding and Donnelly conclude, correctly on the basis of their assumptions, that the suspect has, when the DNA evidence is included, about a one in three chance of being innocent. Their figure of half a million, however, is an entirely arbitrary choice, and is not relevant to real cases where DNA profiling has been used as evidence. In a typical case, a match arises because a suspect's DNA has been profiled and compared to that from a DNA sample from the scene of a crime. Forensic scientists have better things to do with their time than to screen systematically, with DNA profiling, individuals who have only a one in half a million chance of being guilty of the crime that is being investigated.

In the future it may be true that suspects emerge through the screening of databases containing very many individuals, whose profiles were not collected in connection with the crime being investigated. In these circumstances it may indeed be true that suspects have very low prior probabilities of guilt, and this should be incorporated in the standard way using Bayes' formula in the assessment of the final probability of guilt.

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SIR — The account by Balding and Donnelly<sup>1</sup> of the use of DNA evidence in courts cannot be correctly accessed from the citations given by the authors. As of 18 March 1994, DNA evidence has been considered in 139 cases in US courts and admitted in 124 of them. Some of the remands or exclusions of the remaining 15 cases are also under re-examination. In no case was DNA the sole evidence implicating the suspect in the crime, unlike the situation depicted by Balding and Donnelly.

The authors' concern about 'false match' errors does not acknowledge that most forensic case analyses provide sufficient scope to check whether errors could

have occurred because the band intensity, size and other attributes of autoradiographs are indicative enough of which sample is the evidentiary one and which bands come from a relatively fresh sample. Furthermore, opportunities for re-testing the samples from suspect, victim and evidence materials have never been used to document 'false match' errors.

I do not suggest that forensic laboratories cannot and do not make mistakes, but reviews of databases generated by forensic laboratories suggest that a match beyond five loci is virtually improbable for any cosmopolitan population<sup>2</sup>, and even in a highly inbred population a multilocus DNA match between close relatives is an exception rather than the rule<sup>3</sup>. For example, in reviewing more than 60 DNA evidence cases, I found that the general forensic laboratories produce autoradiographs that meet current scientific standards. In no two cases are the profiles even close to each other, and in every case no individual was found in a large database (more than 10,000 individual records) with a matching profile. A simple probability calculation would show that this cannot result from DNA profiles with 'unsafe' small reported frequencies.

Rhetorical criticisms of DNA testing can only generate confusion among judges and jurors alike. Significant effects of population substructure or not, the rarity of any specific hypervariable multilocus DNA profile is a biological fact. Whether or not a relative of the suspect is the perpetrator can be examined with a simple test without statistical arguments such as discussed by Balding and Donnelly.

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SIR — Balding and Donnelly<sup>1</sup> consider that courts have been misled about the strength of DNA evidence. But although they make the correct distinction between the probability that a particular individual has the DNA profile in question and the probability that he has this profile given that the defendant has it, they overstate the distinction. For a single locus with allelic frequencies of 0.1 (their figures), the expected frequency of any heterozygote is 0.02 while the conditional frequency in a population for which the measure

of population structure  $F_{ST} = 0.001$  is the very similar 0.0203 (ref. 4). The difference between their figures of  $10^{-7}$  and "might be  $10^{-5}$  or more" corresponds to an  $F_{ST}$  of 0.05 or more. It is clear that 0.001 is a much more realistic value than 0.05 (refs 5, 6).

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## Optimism abounds

SIR — In commenting on Kay Davies's decision not to take up her post as director of the Medical Research Council (MRC)'s new Clinical Sciences Centre (CSC), you reported a number of comments casting doubt on the future of research in the new Hammersmith Hospitals Trust (*Nature* 369, 92; 1994).

Although we are disappointed at Davies's decision, we remain optimistic about the future of research in the new trust. In particular:

■ The MRC remains fully committed to the CSC, a replacement for Kay Davies is now being sought and the new laboratory building opens in June.

■ The Royal Postgraduate Medical School, which has attracted the highest ratings in recent reviews by the Higher Education Funding Council for England, the Department of Health and so on, remains at Hammersmith Hospital.

■ The government's formula for funding the excess costs of postgraduate research is satisfactory.

■ The new trust, one of the largest in the National Health Service, is the dominant service provider in west London, and has a powerful concentration of specialist services that will provide the patient flow required to sustain high quality research. The trust had adopted the academically led clinical directorate structure, which was one of the foundations of Hammersmith's success.

■ The combination of Charing Cross with Hammersmith has brought additional strength in several important areas, notably neurosciences, cancer and rheumatology.

For these reasons we do not share the pessimistic tone of your article. The new Hammersmith Trust, with its associated postgraduate and undergraduate medical schools and institutes, is already a powerful force in research, service and teaching. Now the uncertainty about the site is behind us, we can build for the future.

**Christopher Bland**  
(Chairman, Hammersmith Hospitals Trust)  
**Colin Dollery**  
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1. Balding, D. J. & Donnelly, P. *Nature* **368**, 285–286 (1994).
2. Risch, N. J. & Devlin, B. *Science* **255**, 717–720 (1992).
3. Chakraborty, R. & Jin, L. *Hum. Biol.* **65**, 875–895 (1993).
4. Cockerham, C. C. *Genetics* **69**, 235–246 (1971).
5. Brookfield, J. *Heredity* **69**, 91–100 (1992).
6. Morton, N. E. *Proc. natn. Acad. Sci. U.S.A.* **89**, 2556–2560 (1992).



# Saving the tiger in the wild

SIR — Until recently, the major threat to wild tiger populations was declining habitat. Operation Tiger, started in 1973, has been experiencing some success in reducing this threat by creating tiger reserves throughout India and Nepal. However, since 1991, poaching (to provide bones for traditional Chinese medicine) has increased dramatically, to the point where persistence of wild tiger populations may once more be in question. According to a recent report, China exported more than 15,000 cartons of tablets, 5,000 kg of confections, and 31,000 bottles of wine containing tiger bone as an ingredient in 1991 alone. Because few wild tigers remain in China, most bone is imported, primarily from the Indian subcontinent.

Anecdotal information indicates the seriousness of this new threat. It is important, however, to provide a quantitative basis for strong policy action. We therefore created a tiger simulation model to explore the effects of poaching on population viability. The model is an individual-based stochastic spatial model, developed according to the extensive data set from Royal Chitwan National Park, Nepal. Our initial results suggest that, as poaching continues over time, the probability of population extinction increases sigmoidally; a critical zone exists where a small incremental increase in poaching greatly increases the probability of extinction.

Tiger poaching has been a worldwide phenomenon for about three years (1991–94). We found that poaching for this length of time generally results in low probabilities of extinction, except at very high levels of poaching (15 tigers a year). This high level of poaching may have occurred at Ranthambhore Tiger Reserve in India where the population is said to have declined from 44–46 tigers in 1991 to 15–20 tigers in 1992. In our model, this level of poaching leads to a 70–90 per cent probability of extinction.

The next three years (1994–97), however, will be critical for tiger conservation, especially for smaller populations. Approximately 95 per cent of the remaining tiger populations throughout the world consist of fewer than 120 tigers. For these populations, three additional years of a low level of poaching (5 tigers a year) leads to a probability of extinction of less than 5 per cent, but if poaching has been and continues to be moderate (10 tigers a year), the probability of extinction is more than 95 per cent. This illustrates dramatically how a small increase in poaching can lead to a much greater probability of extinction. Given the dire consequences of moderate to high levels of poaching and the relative ease with which tigers are poached, it is imperative that we act quickly to strengthen the already con-

siderable efforts to reduce poaching.

Successful anti-poaching efforts must include a systematic and objective method to assess accurately the size and the geographic distribution of individual tiger populations. After 20 years of research and management under Operation Tiger, the sizes of individual populations and the extent of contiguous habitat that they occupy are not known anywhere except in Nepal. Tiger habitat distribution maps combined with local density estimates would be an objective method to estimate individual population sizes. A coordinated international effort is once again essential to save the tiger in the wild.

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## Vatican error

SIR — You report (*Nature* 368, 90; 1994) the Vatican's announcement that the Pope intends to create a Pontifical Academy for Life to provide counsel on ethical matters concerning biology and medicine. The concept of such an institution, consisting of 70 members to be nominated by the pontiff, appears noble and worthwhile, but the requirement that each member must embrace the view that "life begins at the moment of conception" is problematic.

Inasmuch as the process of conception requires the fertilization of a living ovum by a living sperm, no *de novo* life arises from the process of conception. Rather, life exists before conception (in each of the individual germ cells), it exists during the process of a successful conception and it continues after conception, barring naturally occurring or artificially induced abortion.

To say that "life begins at the moment of conception" is quite different from supporting the view that "life exists before, during and after the moment of conception". The first statement posits "spontaneous creation of life" to explain how such a phenomenon could occur while the latter statement does not.

It would be a shame if competent nominees for the Pontifical Academy for Life were screened out in favour of less qualified ones, but it seems that a poten-

tially worthy and important new edifice is being constructed on a defective foundation.

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## Early astronomy

SIR — Ziauddin Sardar, in reviewing Toby Huff's *The Rise of Early Modern Science: Islam, China and the West* (*Nature* 368, 376–378; 1994), suggests that the Dogon knew many astronomical observations 1,500 years earlier than Galileo. He claims that they pose problems for an "emerging new strain of deep Eurocentrism".

They do not, as they never existed. The 'Dogon astronomical observations' are a case of misanthropology like that propounded by Margaret Mead. Their originator was Marcel Griaule, a French anthropologist who had studied astronomy in Paris. Griaule was strongly 'anti-eurocentric', seeking evidence in Africa to show that non-European societies had produced cultures with thought as sophisticated as that of Europe. And, of course, he 'found' it. While he was studying Dogon secret societies, his informants picked up on his interest in astronomy and told him what he wanted to hear. All was exposed by W. Van Beek a few years ago in *Current Anthropology* (32, 139–167; 1991).

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## Eastward in Eden

SIR — I noted with interest your photograph of *Amorphophallus titanum* at "a botanical garden" in Indonesia (*Nature* 368, 399; 1994).

The garden in question is Bogor Kebun Raya, founded in 1817, which is internationally famous and by far the best of the botanical gardens in South-East Asia. This garden and its diligent staff are relatively poorly funded by the environmentally unsympathetic Indonesian government and the garden is frequently under threat of development. It is therefore a pity to waste an opportunity to advertise its importance.

If this spectacular flower had been blooming in Kew Gardens, would you have said "in a botanical garden in London"? I doubt it.

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# Making publication more respectable

**The benefits of weakening the link between publication and personal success would be immense, and the place to start is with the widespread practice of spurious co-authorship.**

How and when will it be possible to arrange that researchers' personal success (and their families' general comfort) can be separated from their publications records? Last week, this journal argued for a deliberate weakening of the link in the belief that the tendency to misuse the literature, and in particular to use it as a means of acquiring a false reputation as a productive researcher, would thereby be diminished (see *Nature* 369, 261–262; 1994). But the research profession is by now so accustomed to the use of publications as indicators of personal performance that there may be some who do not think the project feasible.

Nothing in what follows, which is meant to demonstrate that a weaker link is feasible as well as desirable, should be mistaken for a diatribe against the process of publication as such. Where, after all, would *Nature* be if people never published written accounts of what they had done? Where, for that matter, would science itself be? A discovery, after all, is no better and no worse than the written account of it eventually appearing in the literature (some of which may well be electronic soon).

But the case for a weaker link is not simply to diminish the incentive for outrageous fraud. On the contrary, the outright fabrication of data that are then woven into a convincing tale, which is almost always a lonely business exercised in seclusion from potentially critical colleagues, would probably continue. (Vanity is a powerful force.) But imaginative people of that kind would not so easily win the patronage of more senior people who are themselves genuinely too busy for research, but who are too proud to say so and who dare, as a consequence, to run the risks to their reputations of co-authorship in work they do not understand.

Indeed, the general interest is that honorary co-authorship as such should be reduced, and not simply for the convenience of the managers of the databases now storing all the names in the literature. For historical purposes, as well as to know who did what, it is important that the authors of publications should in some meaningful sense be jointly and severally responsible for the content of what is published. Can that be the case when the number of the authors of an account of a particle accelerator experiment may easily exceed a hundred or two?

To single out these extremes is not to suggest that the majority of the authors have been sleeping partners. In the nature of accelerator experiments, most of the army

of authors will have made vital, but often very particular, contributions to the joint enterprise. But the inclusion of their names in such a list of authors does not help users of the literature to appreciate what they have done, and how well, while their prospects of personal advancement will depend almost exclusively on the opinions formed by group leaders and other senior people of their work. In other words, in these extreme cases, a person's appearance in a list of authors hardly matters, but his colleagues' appraisal does.

As it happens, co-authorship is as serious an anxiety in biology. Does it, for example, make sense that physicians who have collected blood from their patients for some collaborative study should appear as authors of the account eventually published of it? Or what is the proper form of acknowledgement for assistance when a person has complied with the conventions of the research trade, and has supplied others with some essential research material? Some journals (but not this one) make it an absolute condition of publication that research materials used will be made available to all serious enquirers. What do they have to say about the practice of suppliers who insist on co-authorship as a *quid pro quo*?

Of course, a person who has supplied some material essential for an investigation, a DNA clone containing a novel gene perhaps, may claim to have made an essential contribution, but if the material arose in the course of an investigation already published, is it necessary, let alone equitable, that he or she should have a second bite at the cherry of credit? Those who look to the published record for a measure of people's productivity as researchers may be dismayed to learn that personal lustre can be a matter for crude negotiation along these lines.

Worse can happen. One of *Nature*'s authors has recently been downcast to learn that two people who had kindly helped to supply the material that was the subject of an investigation had less kindly supplied the same material to a larger group elsewhere, but without disclosing that circumstance. The result will be two nearly simultaneous publications in two different journals, with (probably) two sets of co-authorship for the zealous suppliers. It is not difficult to guess what this kind of happening does for the civility of the research enterprise.

It is more difficult to decide on a workable antidote for the abuse of co-authorship. The principle is that every listed author should at least be able to give a brief talk at a public meeting on the substance of what

had been reported in writing. He or she would be forgiven if some of the questions raised in the discussion required the presence of a colleague specialized in one or other of the specialized techniques involved, but not for failing to describe the antecedents of the work or to give a coherent account of the result and its importance.

Sadly, there are no easy written tests of this distribution of responsibility among the authors of manuscripts submitted for publication. The benefits of authorship are so great that potential authors of manuscripts awaiting publication would probably, without hesitation, sign formal declarations that they had been full members of the group responsible. And the prospects are slender of carrying through a scheme advocated some years ago by some editors of journals that credit should be apportioned among authors by decimal fractions adding up to 1.0 for each published article. A better first step would be that authors themselves should include a brief statement in their text of who did what when a piece of work has involved the collaboration of people with different skills.

Ridding the literature of spurious co-authorship is regrettably only a small part of what needs to be done if the literature is to become a record of discovery, not just a *curriculum vitae* for every working scientist. But there are some lessons to be learned from even a cursory consideration of that task.

Not the least of them is the recognition that, among the small armies of people who dance attendance on particle accelerator experiments, the presence of a name among the many authors of a publication can only be an unhelpful guide to that person's quality and potential as a researcher, and that the opinions of others on these points must be crucial to that person's career. There is much to be said for extending that practice more widely, to fields in which the difficulty of knowing who did what, and how well, is not the mere number of authors at the head of a publication, but the certain presence therein of spurious co-authors.

The obvious danger is that nepotism or even prejudice may then have an influence, but one of the benefits of the present universal competition for research funds is that only the short-sighted would fail to resist those influences. In short, the disappearance of spurious co-authors would help enormously in dealing with the appointment and promotion of people to research posts. The competition for research grants is a more difficult problem. **John Maddox**



# Origins revealed in demise

Nino Panagia

THE ring around supernova 1987A in the Large Magellanic Cloud, shown in the image below, has puzzled astronomers since its very detection, and its nature remains controversial. On page 378 of this issue<sup>1</sup>, McCray and Lin put forward the fascinating hypothesis that it may be the remnant of the primordial cloud out of which the supernova progenitor formed about 10 million years ago.

Evidence for copious material surrounding the supernova first surfaced in late May 1987, about three months after the explosion was first seen. Very narrow emission lines of highly ionized species such as carbon, nitrogen and oxygen were detected<sup>2</sup>, their narrow widths and the overabundance of nitrogen indicating that the emitting gas consisted of matter ejected by the supernova progenitor in its red supergiant stage. (The progenitor, the star Sk-69° 202, had a complex history, of which more later.)

Then came the news that the material was not arranged in a nice spherical shell, as theorists like to assume for their models. The notion first came from observations made by Crotts *et al.*<sup>3</sup> at the Cerro Tololo Interamerican Observatory, which revealed an elliptical

structure about two arcseconds across and emitting mostly emission-line radiation (H $\alpha$  and the [O III] line at 500.7 nm). Subsequent, higher-resolution observations made with the ESO New Technology Telescope at La Silla<sup>4</sup> confirmed the shape and the intensity of the elliptical structure and revealed further details, including outer loops that resembled the inner ellipse but were more extended and much fainter.

It took the resolving power of the Hubble Space Telescope in August 1990 to determine unequivocally that the bright ellipse was a genuine ring and not the bright rim of a thin elliptical shell. And combining the accurately measured size and shape of the elliptical structure with measurements of its absolute diameter from light travel times demonstrated<sup>5</sup> that the observed ellipse was an almost perfectly circular ring inclined by about 43° to the line of sight.

The presence of a dense, thin ring surrounding a massive star at the end of its evolution is not easy to account for. On the one hand, the ring consists of material processed through the C, N, O cycle that

should have been ejected in the form of a slow stellar wind (moving at about 10–20 km s<sup>-1</sup>) by the progenitor when it was in its red supergiant phase. On the other hand, in the last few thousand years before exploding the progenitor underwent a final evolutionary step, shrinking and heating up to become a blue super-

polar mass loss rate<sup>6</sup>. Second, it is difficult to see why the expansion velocity of the ring is so low, if it results from compression by a fast wind.

McCray and Lin's alternative picture circumvents most of the objections, and, as a bonus, can be tested by future observations. Their idea is that around the supernova lies a dense protostellar disk, left over after the formation of Sk-69° 202. They show that this disk could easily have had enough mass to stop the stellar wind and the correct size to account for the ring's diameter. In this model, the ring should actually be the bright inner rim of a larger, fairly massive disk that at present is mostly neutral and, as such, is not readily detectable. The inner rim itself can be seen because it has been heated and ionized by the intense ultraviolet radiation produced in the supernova explosion.

Of course, it may look like they succeed where others run into difficulties simply because they add another ingredient. But this ingredient is not really new at all. It is just what might have been left behind from the process that formed the progenitor star. Historians may like to note that Meikle *et al.*<sup>7</sup> first put forward the hypothesis of a protostellar disk around SN1987A in 1990, suggesting that it would account for the low expansion of the ring; they limited themselves to a qualitative statement, however, without calculating the effects in detail.

The experimental test of this hypothesis is a few years away yet. Around the year 2000, the supernova ejecta will reach the ring and will collisionally heat and excite it, making it brighter by orders of magnitude at optical, ultraviolet and X-ray wavelengths (see, for example, ref. 8). Now, if the ring is just an isolated ring, then the interaction will last a few years before the bulk of the ejecta reaches it and sweeps it away. But if McCray and Lin's suggestion is correct, the substantial mass of the protostellar ring will allow it to withstand the impact of the ejecta for longer and to become more luminous. It is just a matter of being patient: the quality

IMAGE  
UNAVAILABLE  
FOR COPYRIGHT  
REASONS

A vivid image of supernova 1987A, taken in visible light by the newly repaired Hubble Space Telescope this February. The bright inner ring discussed by McCray and Lin on page 378 of this issue is clearly visible, as are two more tenuous outer rings whose origin is still more mysterious.

giant. As such, it should have been emitting a fast wind (about 500 km s<sup>-1</sup>).

The current hypothesis, then, is that the ring was created when the blue supergiant wind caught up the material from the red supergiant phase. To create a dense ring rather than a shell, one has to argue that the material ejected in the red supergiant phase was denser and slower in the equatorial plane than along the polar axis; the blue supergiant wind formed the ring by compressing this denser material. In the polar directions the wind expanded more freely and, by interaction with the slower red supergiant wind, created a peanut-shaped bubble whose length is several times the diameter of the ring. In other words, the dense ring is a sort of thick belt around the waist of a larger bubble.

This explanation, although 'natural' in the sense that it uses ingredients taken directly or indirectly from observational evidence, has a couple of unsatisfactory points. First, to obtain a disk as thin as observed, one has to assume a very anisotropic wind in the red supergiant phase, with a high ratio of equatorial to

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1. McCray, R. & Lin, D. N. C. *Nature* **369**, 378–380 (1994).
2. Wamsteker, W. *et al.* *IAU Circ. No.* 4410 (1987).
3. Crotts, A. P. S., Kunkel, W. E. & McCarthy, P. J. *Astrophys. J.* **347**, L61–64 (1989).
4. Wampler, E. J. *et al.* *Astrophys. J.* **362**, L13–16 (1990).
5. Panagia, N. *et al.* *Astrophys. J.* **380**, L23–26 (1991).
6. Luo, D. & McCray, R. *Astrophys. J.* **379**, 659–662 (1991).
7. Meikle, W. P. S., Cumming, R. J., Spyromilio, J., Allen, D. A. & Mobasher, B. *ESO/EIPC Workshop on SN 1987A and other Supernovae* (eds Danziger, I. P. & Kjær, K.) 595–603 (ESO, Garching bei München, 1991).
8. Wang, L. & Wampler, E. J. *Astr. Astrophys.* **262**, L9–12 (1992).

and the duration of the fireworks will tell us which is the right answer.

Why do we care? In part, of course, it is pure curiosity about supernovae and the evolution of massive stars. But that is not the only reason. If protostellar disks around evolved massive stars are common, then observations of old supernovae should let us see a rekindling of the fire as expected for SN1987A. And if we could establish a quantitative relationship be-

tween the nature of the progenitor and the properties of the remnant protostellar disk, we could be granted another way to determine cosmological distances and clarify the really hot problem of the distance scale of the Universe. □

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## IMMUNOLOGY

# Promethean viruses?

*Paul M. Allen and Rolf M. Zinkernagel*

VIRUSES have come up with many ways to avoid recognition and elimination by host antibodies or T cells. They include integration into the host genome, hiding from or blocking the immune system, changing their antigens, or use of several of these techniques in concert (as is the case with the human immunodeficiency virus, HIV)<sup>1,2</sup>. Klenerman *et al.*<sup>3</sup> and Bertoletti *et al.*<sup>4</sup> (pages 403 and 407 of this issue) now provide evidence of a further, more sophisticated strategy — this is antagonism, whereby a virus can combat antiviral effector T cells directly by blocking their activity. The two groups show that in HIV-1 and hepatitis B virus (HBV) infections, naturally occurring variants of the epitopes recognized by cytotoxic T lymphocytes (CTL) may act as antagonists *in vivo*, because the corresponding peptides specifically prevent a CTL response directed against the virus *in vitro*.

Through their antigen-specific receptor, T cells recognize a ligand composed of a peptide fragment of an antigen bound to a molecule of the major histocompatibility complex (MHC). The T-cell receptor (TCR) can interact with non-stimulatory variants of the normal ligand, but this results in T-cell inactivation (reviewed in refs 5–7). These variant peptides contain a substitution in an amino-acid residue contacted by the TCR, but can bind normally to MHC molecules. The effects of these variant peptides include the positive selection of thymocytes, induction of some but not all T-cell functions (for example cytokine production without proliferation), anergy induction and TCR antagonism. TCR antagonism occurs when variant peptides are co-incubated with an agonist (wild-type peptide), resulting in a dose-dependent inhibition of the T-cell response. TCR antagonism was first demonstrated for human CD4<sup>+</sup> T cells<sup>7</sup>, and subsequently for a class I restricted CD8<sup>+</sup> CTL response<sup>8</sup>.

McMichael and colleagues previously identified strong CTL epitopes of HIV-1 Gag protein in asymptomatic infected individuals and isolated naturally occur-

ring variants of these epitopes. In the new study<sup>3</sup> they examined these variant peptides and demonstrated that they can act as TCR antagonists for the *in vitro* CTL response against the original CTL epitopes. This phenomenon was shown for three different epitopes from several individuals, and for a consensus variant identified from the HIV sequence database. Importantly, these variant epitopes can act as antagonists not only in the form of exogenous peptides but also when expressed as proteins by infectious vaccinia recombinant virus.

Ferrari and colleagues<sup>4</sup> report similar findings from their studies of the CTL response to a nucleocapsid epitope in two HLA-A2-expressing individuals chronically infected with HBV. Both people were found to be homogeneously infected with HBV variants which contained two substitutions in the nucleocapsid epitope compared to the index sequence. This variant epitope also antagonized the CTL response to the wild-type epitope. These antagonists are very potent, and function after being synthesized in the target cell or as an exogenous peptide.

In both studies, the presence of the antagonist prevents the T cell from performing its effector function, but does not directly turn it off, a phenomenon previously reported in Th1 clones after stimulation with analogue peptides alone<sup>9</sup>. All previous reports of TCR antagonists required a molar excess (10–100 times) of antagonist over agonist, however. The antagonists described by Klenerman *et al.* and Bertoletti *et al.* were much more active, being able to inhibit the response at antagonist:agonist ratios of 0.01:1 to 0.1:1. This increased effectiveness could simply be due to the inherent activity of the variant peptides and because they were selected *in vivo*. The way in which TCR antagonists function is not yet known, but several, non-mutually exclusive, mechanisms have been proposed<sup>5,7</sup>. One proposal is that the non-stimulatory ligands play a passive role, diluting out stimulatory ligands;

another is that antagonists actively deliver a negative signal upon TCR contact. The identification of highly active antagonists would seem to favour the active model.

The new findings suggest that a promethean approach is involved in the selection process of viruses such as HIV and HBV which favours these antagonistic variants (Prometheus looked forward, and stole fire from the gods for humanity); this contrasts with the usually accepted epimethean means of avoiding immunity (brother Epimetheus was always a step behind). Cytopathic viruses that destroy the cells they infect, using the antagonist approach, would be doomed because they would kill the host even more efficiently than usual. But for a non-cytopathic virus, impairing or even turning off antiviral CTLs could exhaust them and be an efficient way to prevent death of the host (and therefore also of the virus) by reducing lethal CD8<sup>+</sup> T-cell-mediated immunopathology<sup>10</sup>. In the case of HIV infection, the infected target cells are CD4<sup>+</sup> T cells and macrophages, whereas for HBV they are hepatocytes.

All this leaves several questions. First, there is usually more than one T-cell epitope per virus and each epitope can be recognized by several T-cell receptors of varying avidities. For each epitope, the virus would have to develop variants which can antagonize all reacting T cells. In an antiviral response restricted to a few epitopes, or during latency when only a few epitopes are expressed, one can imagine that the antagonistic variants would be more effective. Also, the antagonists themselves should be immunogenic to a new population of CTL, leaving only a limited time for these variants to be effective before the cells they have infected are killed by the new CTL.

Second, these events have to occur in a sea of agonistic viruses. How could antagonistic ones become so effective, and in some cases dominant? Perhaps the antagonists act synergistically with other viral escape mechanisms. For the actual infected cell in which a variant virus is generated, the presence of an antagonist would be beneficial because it would allow both the wild-type and variant viruses to

1. Mims, C. A. *The Pathogenesis of Infectious Disease* (Academic, London, 1982).
2. Zinkernagel, R. M. & Hengartner, H. *Nature* **354**, 433–434 (1991).
3. Klenerman, P. *et al.* *Nature* **369**, 403–407 (1994).
4. Bertoletti, A. *et al.* *Nature* **369**, 407–410 (1994).
5. Evald, B. D., Sloan-Lancaster, J. & Allen, P. M. *Immun. Today* **14**, 602–609 (1993).
6. Marrack, P. & Parker, D. C. *Nature* **368**, 397–398 (1994).
7. Sette, A. *et al.* *Rev. Immun.* **12**, 413–431 (1994).
8. Jameson, S. C., Carbone, F. R. & Bevan, M. J. *J. exp. Med.* **177**, 1541–1550 (1993).
9. Sloan-Lancaster, J., Evald, B. D. & Allen, P. M. *Nature* **363**, 156–159 (1993).
10. Moskopidis, D., Lechner, F., Pircher, H. & Zinkernagel, R. M. *Nature* **362**, 758–761 (1993).
11. Odermatt, B., Eppler, M., Leist, T. P., Hengartner, H. & Zinkernagel, R. M. *Proc. natn. Acad. Sci. U.S.A.* **88**, 8252–8256 (1991).
12. Peters, M. *et al.* *Hepatology* **13**, 977–994 (1991).



survive and replicate. The variant virus, once it has replicated, could further mutate, replicate or be selected to avoid immune recognition; alternatively, if the original variant virus has additional growth advantages it will win out.

From these considerations, it follows that only non-cytopathic viruses can establish chronic carrier states by using the antagonistic approach. The results of these studies could therefore support the hypothesis that HIV is non-cyto-

pathic *in vivo*, and that AIDS is therefore a consequence of T-cell-associated immunopathology<sup>2,11</sup>, as is already well established for hepatitis caused by HBV<sup>12</sup>. □

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## METEORITICS

# Martians come out of the closet

Monica M. Grady and Ian P. Wright

AT about the time that scientists and engineers were coming to terms with the loss of the Mars Observer spacecraft, a new and as yet unique sample of the martian crust was discovered on Earth. The identification of an unusual meteorite always creates a stir within the meteoritics community; when the find turns out to be a piece of Mars, then the excitement has an even greater edge. In a report in *Meteoritics*, D. W. Mittlefehldt<sup>1</sup> describes the reassignment of ALH84001 (a meteorite from the Allan Hills region of Antarctica) from diogenite, a relatively common type of basalt from the asteroid belt, to an SNC-related meteorite — in other words, probably from Mars. The affair has forced scientists not only to re-examine the criteria by which meteorites are pigeonholed into the current fairly rigid classification scheme, but also to re-evaluate models of petrogenesis on Mars, and, most particularly, to look again at hydrothermal processes which may have modified the martian crust.

One of the reasons that ALH84001 was not immediately recognized as an SNC meteorite is that it does not fit readily into either of the established subdivisions, shergottite (S) or nakhlite-Chassigny (NC). Instead, it represents a new class of martian material. Its dominant mineralogy is a homogeneous orthopyroxene, with minor chromite, maskelynite, apatite, pyrite and carbonate. The melt composition inferred from its mineralogy is intermediate between those calculated for the shergottites and the NC clan. ALH84001 fits into neither group, but possesses features common to both, implying that it forms an important link between the subdivisions.

The most immediately obvious accessory mineral phase in ALH84001 is a bright orange-coloured iron-magnesium-calcium carbonate, containing minor amounts of manganese. The carbonates are present in two forms: as large grains up to 200 µm across with complex, fine-scale compositional zoning, or as small single

grains about 10 µm in size, which are unzoned. The chemical analysis of the carbonates allows comparison to be made with terrestrial carbonates, and from this Mittlefehldt proposed that the carbonates in ALH84001 were formed by multiple influxes of fluid at temperatures possibly as high as 700 °C. In other words, the new martian rock gives us evidence of a hydrothermal system on another planet.

The mineralogy of ALH84001 will undoubtedly help in working out martian geochemical cycles. Mars specialists have long maintained that carbonate reservoirs must exist on the planet, formed by interaction of the CO<sub>2</sub>-rich atmosphere with loose soil and ash covering the bedrock<sup>2</sup>. But there has been only poor evidence for these deposits — no samples have been gathered directly from Mars, and spectral data are ambiguous. In contrast, carbonates are readily observed in SNC meteorites: Carr *et al.*<sup>3</sup> were the first to infer the presence of carbonates in Nakhla and EETA79001 (a shergottite) from acid-dissolution experiments. They were followed by Gooding *et al.*<sup>4</sup>, who found carbonate deposits (mainly calcite) in the small cavities known as 'vugs' in EETA79001.

Subsequent work has shown that almost all the SNC meteorites contain carbonates<sup>5</sup>, but never on the scale and abundance observed by Mittlefehldt. The Fe,Mg-rich composition is also distinct from that of other martian carbonates, which are mainly calcite; perhaps this implies that carbonates in ALH84001 had a different mode of formation.

The origin of carbonates in SNCs is an open question. As the highest concentrations have been identified in the Antarctic shergottite EETA79001, it has been suggested that they are simply Antarctic weathering products. Radiocarbon and stable carbon and oxygen isotope data have not always been straightforward to interpret. In contrast, the carbonate history of ALH84001 appears straightforward: carbonates that formed early in the

history of the parent rock are coarse-grained and strongly zoned, from Fe- and Ca-rich endmembers to almost pure magnesite. These assemblages are frequently cut by fractures, along which carbonate grains are often displaced, indicating that they formed before a shock event cracked the rock. Together with the inferred high formation temperature, this is strong evidence that the carbonates are indigenous to ALH84001, and are not simply terrestrial weathering products.

A second generation of unzoned carbonates occurs within regions of crushed material. These grains are smaller than the zoned carbonates but have the same general composition, so they were probably formed by remobilization of early carbonates during subsequent shock events.

How could the carbonates have formed? Textural and mineralogical evidence indicate that formation conditions varied: the carbonates might have precipitated from a single fluid whose composition changed during cooling, or the ambient temperature might have remained roughly constant during multiple influxes of different fluids. We must look to analyses of the carbon and oxygen stable isotope compositions to distinguish between these possibilities. Material has already been distributed to three laboratories for analysis, and results are awaited with interest.

It has become increasingly apparent that a direct mission to collect Mars samples is unlikely this century, and possibly far into the next. The failure of Mars Observer and uncertainty over the fate of Mars 94 and Mars 96 have contributed to the general malaise surrounding martian science. We must therefore grasp every opportunity to study our neighbouring planet, and once again the Antarctic meteorite collection programme has delivered an invaluable sample to the scientific community. Meteoriticists have long delayed in unanimously recognizing Mars as the SNC parent body. As Mittlefehldt concludes in his paper: "It is time to come out of the closet and openly refer to those meteorites . . . as martian". Samples of Mars are available for study — and the recent recognition of ALH84001 shows that there is much worth studying. □

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1. Mittlefehldt, D. W. *Meteoritics* **29**, 214–221 (1994).

2. Kahn, R. *Icarus* **62**, 175–190 (1985).

3. Carr, R. H., Grady, M. M., Wright, I. P. & Pillinger, C. T. *Nature* **314**, 248–250 (1985).

4. Gooding, J. L., Wentworth, S. J. & Zolensky, M. E. *Geochim. cosmochim. Acta* **52**, 909–915 (1988).

5. Wright, I. P., Grady, M. M. & Pillinger, C. T. *Geochim. cosmochim. Acta* **56**, 817–826 (1992).

# A new look for the 1990s

Drew M. Pardoll

THIS is emerging as a banner year for tumour antigens, the latest evidence coming in the form of papers by Mandelboim *et al.*<sup>1</sup> and Cox *et al.*<sup>2</sup>. These two groups report the first successful identification, using strictly biochemical approaches, of tumour peptide antigens restricted by class I molecules of the major histocompatibility complex (MHC). Not only does this and related work bear on our understanding of how the immune system interacts with tumour cells, it also has implications for the development of antigen-specific immunotherapies.

Tumour antigens have a new look in the 1990s, one which stems from a change in ways of fishing for them. In the 1980s antibodies were the hook, but these days immunologists are hunting with T cells. T cells are the critical mediators of tumour specificity in developing adoptive immunotherapy and cancer vaccines, which makes sense in the light of how peptides derived from proteins in any cellular compartment can associate with MHC class I molecules to be presented to CD8<sup>+</sup> (cytotoxic) T cells. Consequently, the universe of potential tumour antigens recognizable by T cells is vast.

Without any prior clues, the task of identifying these antigens is technically daunting. The T-cell receptor (TCR) cannot be used as a reagent for affinity purification, because only a tiny fraction of MHC molecules will be occupied by the cognate peptide, and because the affinity of the TCR for its peptide-MHC complex is low. To circumvent this roadblock, Boon and colleagues<sup>3,4</sup> drew a page from bacterial genetics in developing the first standardized strategy for cloning genes encoding tumour antigens recognized by CD8<sup>+</sup> T cells. Once the target gene has been identified, the region encoding the epitope can be narrowed down and ultimately candidate peptides are synthesized to determine which most effectively stimulates the tumour-specific T cell. This approach has been used<sup>5-11</sup> to net a fistful of new human melanoma antigens (of which more below).

The biochemical strategy adopted by Mandelboim *et al.*<sup>1</sup> and Cox *et al.*<sup>2</sup> begins with the standard procedure of acid-eluting peptides bound to MHC class I from tumour cells, followed by fractionation with reversed-phase high-performance liquid chromatography (HPLC)<sup>12,13</sup>. Bioactive peptides recognized by the tumour-specific CTL are identified by adding the HPLC fractions to an antigen-processing mutant predominantly expressing empty class I molecules on the surface. Free peptides bind to the empty molecules so that fractions that

sensitize the surrogate target for lysis by the tumour-specific T cells can be identified, further purified and ultimately sequenced. This approach is not for the faint-hearted — the huge number of different MHC class I-associated peptides (some 10,000–50,000 per cell) makes the task of identifying the single cognate peptide a biochemist's version of finding a needle in a haystack.

fractionation to identify a shared, HLA-A2-restricted melanoma peptide recognized by CTL lines derived from T cells from patients' lymph nodes. Because each of several bioactive fractions contained over 50 peptides on mass spectroscopic (MS) analysis, a second-dimension HPLC fractionation, using different elution conditions, was required. At this point, tandem MS was employed to separate and then sequence a number of peptides in one of the bioactive fractions. The first MS was used to select and transfer individual peptides to the second, in which they were sequenced by a fragmentation technique.

**MHC class I-restricted tumour antigens**

| Tumour                      | Antigen     | Normal adult tissue distribution | Genetically altered in tumour? | Source of T cells                                             |
|-----------------------------|-------------|----------------------------------|--------------------------------|---------------------------------------------------------------|
| Murine P815 mastocytoma     | P1A         | Testes                           | No                             | Vaccinated mice                                               |
| Murine Lewis lung carcinoma | Connexin 37 | Lungs                            | Yes*                           | Vaccinated mice                                               |
| Human melanoma              | MAGE-1      | Testes                           | No                             | Peripheral blood lymphocytes from vaccinated patient          |
| Human melanoma              | MAGE-3      | Testes                           | No                             | Peripheral blood lymphocytes from vaccinated patient          |
| Human melanoma              | MART 1/Aa   | Melanocytes                      | No                             | Peripheral blood lymphocytes, tumour-infiltrating lymphocytes |
| Human melanoma              | gp100       | Melanocytes                      | No                             | Tumour-infiltrating lymphocytes, draining lymph node          |
| Human melanoma              | Tyrosinase  | Melanocytes                      | No                             | Peripheral blood lymphocytes, tumour-infiltrating lymphocytes |

\*Unusual genetic alteration consisting of three consecutive nucleotide substitutions.

Mandelboim *et al.*<sup>1</sup> had the simpler task, probably because their tumour antigen represented an exceptionally large needle. They used bulk CTL cultures to identify a tumour antigen from the spontaneously arising murine Lewis lung carcinoma. This line was produced by immunizing mice with a whole-cell vaccine genetically engineered to express increased levels of an autologous MHC class I molecule. Edman sequencing of a single bioactive fraction turned up a predominant octapeptide sequence which matched that of the gap junction protein, connexin 37, in 7 of 8 positions. In this case, the most highly represented peptide indeed turned out to be the immunologically active peptide. This result indicates that the altered connexin 37 peptide constituted at least 50 per cent of the total material in the bioactive fraction, and is thus quite dominant among the total MHC class I-associated peptides on Lewis lung carcinoma cells.

A more complex picture emerges from the paper of Cox *et al.*<sup>2</sup>, who used peptide

The third peptide sequenced, derived from the melanocyte-specific membrane protein, gp100, reconstituted recognition by melanoma-specific T-cell lines from four separate patients, thereby implicating it as a common, shared melanoma antigen.

At several hundred copies per cell, or roughly 0.1 per cent of total MHC-bound peptide, gp100 is apparently present at a much lower level than the Lewis lung peptide. Interestingly, despite its potency in stimulating T cells, it was estimated to bind relatively poorly to HLA-A2, implying that the affinity of the peptide-A2 complex for TCR is quite high. This result suggests that the peptide is efficiently loaded onto the MHC molecule via the endogenous pathway, despite a relatively high off-rate once bound. Consistent with a report that affinity of the peptide-MHC complex for TCR is an important determinant of T cell reactivity<sup>14</sup>, these findings raise a cautionary flag about simply measuring MHC binding to identify the best epitopes for vaccine generation. ►



Given the lack of cross-reactivity among tumour-specific T-cell lines generated against murine skin tumours induced by ultraviolet light<sup>15</sup>, one might have predicted that most melanoma-reactive T cells would recognize bona fide tumour-specific antigens derived from mutated genes. Indeed, expectations that tumour-specific genetic alterations could produce tumour-specific peptides have launched a number of fishing expeditions for T cells recognizing peptide products of mutated oncogenes or tumour-suppressor genes. Nonetheless, the limitations on peptide binding to MHC class I molecules would restrict the general usefulness of peptide vaccines derived from an oncogene or tumour-suppressor gene.

Known tumour antigens are listed in the table; notably, all were identified without biasing the search towards a specific gene product. As the targets of T cells derived from either vaccinated or tumour-bearing individuals, they probably represent antigens against which tolerance is least stringently maintained. This notion is strengthened by the fact that many of the melanoma antigens were independently identified from different patients using different sources of T cells for the screening.

Some interesting themes emerge from the table. First, none of the antigens arise from the products of known oncogenes or tumour-suppressor genes. The only one stemming from an apparently mutated gene is the connexin 37 peptide in Lewis lung carcinoma. However, the reported Cys→Gln conversion can only be generated by nucleotide substitutions at all three codon positions. Such an alteration is highly unusual, and may instead represent a polymorphism in the mouse in which the tumour originally arose, or an event occurring sometime during the decades of passage *in vitro*.

Remarkably, none of the remainder are tumour-specific neoantigens. Instead, they fall into two categories. Antigens of the P1A and MAGE family are not expressed in any normal adult tissues (with the exception of testes), and are possibly developmental antigens re-expressed during the process of tumorigenesis. Indeed, MAGE-1 can be activated by demethylating agents such as 5-azacytidine; the altered methylation state commonly observed in cancers may thus account for their activation. Its relative tumour specificity makes MAGE-1 an excellent potential vaccine target. Subsequent searches have failed to reveal many patients with MAGE-1 reactive T cells, indicating that it represents a nondominant tumour antigen. The remaining melanoma antigens (tyrosinase, gp100, MART 1/Aa) are differentiation antigens specific to the melanocyte lineage. gp100 and MART 1/Aa seem to be dominant; a single peptide in each is recognized by T cells

from many HLA-A2<sup>+</sup> patients.

Why don't the T cells from these immunized animals or patients recognize tumour-specific neoantigens? The reason may be that when a neoantigen arises within a tumour, it is tolerated by the immune system equivalently to a tissue-specific antigen. So the efficiency with which a particular epitope is processed, presented and ultimately recognized by a TCR is a much more critical determinant of immunological reactivity than is neo-antigenicity. As ubiquitous as genetically altered proteins appear to be in cancer cells, they are still a relatively minor source of total MHC class I-associated peptides. Only if a tumour-specific neo-epitope happens to be effectively processed and presented will it qualify as a dominant target for T-cell immunity. The altered connexin 37 peptide in Lewis lung carcinoma is probably one such example, as shown by its unusually high representation among the peptides bound to MHC class I.

Another determinant of immunogenicity is the tissue type in which the potential antigen is expressed. Experiments with transgenic mice suggest that mechanisms of tolerance to the same antigen can differ depending on which tissue type it is expressed in<sup>16</sup>. It is no coincidence that most known T-cell tumour antigens are derived from human melanoma. This tumour appears to be particularly immunogenic; among human tumours, it is the most sensitive to immunotherapy and is the easiest to generate T cells against. The rules of antigenicity may be quite different for tumours of different histological origin.

The main clinical value of T-cell-defined tumour antigens is in vaccine development. If we can identify targets against which tolerance can most readily be broken, these would probably be the best candidates for generating successful therapies. At first glance, vaccines using antigens that are not truly tumour specific would seem to be inappropriate because of their potential for generating auto-immunity. Often, however, the tissues from which common tumours arise are themselves dispensable. The prostate is probably the best example. In the case of melanoma, some melanocyte-specific antigens are also expressed in certain cells of the retina, inner ear and brain; whereas melanoma patients receiving immunotherapy occasionally develop vitiligo, they do not develop abnormalities of the visual, vestibular, or central nervous system. Also, the normal tissue counterparts of many tumours exhibit extremely low levels of MHC class I, thereby cloaking them from recognition by tissue-specific CTL. So a wide enough window for therapy may occur, even if tolerance is broken against tissue-specific antigens.

As other antigen targets of tumour-

reactive T cells are identified, approaches to generate effective immune responses against them must be evaluated. There has been much interest in peptide vaccines because of their ability to generate CTL responses, though established tumours have yet to be cured with a single peptide vaccine. Also, individual peptides will only have general therapeutic value if they can be efficiently presented by common MHC alleles such as HLA-A2. The finding that some of the melanoma antigens produce several peptide targets in patients with different HLA haplotypes provides a basis for vaccine strategies that use the entire antigen and allow the individual's own MHC alleles to choose the epitope.

Finally, the emphasis on MHC class I-restricted tumour antigens has overshadowed the equally important MHC class II-restricted CD4 response. Adoptive transfer experiments, as well as analyses of genetically modified tumour vaccines, show that CD4<sup>+</sup> cells are every bit as necessary for generating efficient systemic antitumour responses as are CD8<sup>+</sup> cells<sup>17,18</sup>. Recently, a shared MHC class II-restricted melanoma antigen has been identified as tyrosinase<sup>19</sup>. The notion that the same antigen encodes both MHC class I and class II epitopes makes sense, given that both class I and class II tumour antigens appear to be presented by antigen-presenting cells derived from bone marrow during induction of the immune response<sup>20</sup>. Linkage between class I and class II restricted epitopes would therefore produce the most efficient interaction between CD4<sup>+</sup> and CD8<sup>+</sup> T cells, and it would seem that the most effective cancer vaccines will use natural or chimaeric antigens containing both MHC class I and class II epitopes. □

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1. Mandelboim, O. *et al.* *Nature* **369**, 67–71 (1994).
2. Cox, A. L. *et al.* *Science* **264**, 716–719 (1994).
3. De Plaen, E. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **85**, 2274–2281 (1988).
4. Van den Eynde, B., Lethe, B., Van Pel, A., De Plaen, E. & Boon, T. *J. exp. Med.* **173**, 1373–1384 (1991).
5. Vander Bruggen, P. C. *et al.* *Science* **254**, 1643–1650 (1991).
6. Gaughier, B. *et al.* *J. exp. Med.* **179**, 921–930 (1994).
7. Brichard, V. *et al.* *J. exp. Med.* **178**, 489–495 (1993).
8. Coulie, P. *et al.* *J. exp. Med.* (in the press).
9. Bakker, A. B. H. *et al.* *J. exp. Med.* **179**, 1005–1009 (1994).
10. Kawakami, Y. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **91**, 3515–3519 (1994).
11. Kawakami, Y. *et al.* *J. exp. Med.* (in the press).
12. Rotzschke, O. *et al.* *Nature* **348**, 252–254 (1990).
13. Van Bleek, G. & Nathenson, S. *Nature* **348**, 213–215 (1990).
14. Sykuliev, Y., Brunmark, A., Jackson, M., Cohen, R. J. & Peterson, P. A. *Immunity* **1**, 15–22 (1994).
15. Ward, P., Koepfen, H., Hurteau, T. & Schreiber, H. *J. exp. Med.* **170**, 217–232 (1989).
16. Ferber, I. *et al.* *Science* **263**, 674–676 (1994).
17. Greenberg, P. D., Kern, D. E. & Cheever, M. A. *J. exp. Med.* **161**, 1122–1134 (1985).
18. Dranoff, G. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **90**, 3539–3543 (1993).
19. Topalian, S. L. *et al.* *Proc. natn. Acad. Sci. U.S.A.* (in the press).
20. Huang, A. Y. C. *et al.* *Science* **264**, 961–964 (1994).

# Convective convictions

Stephen Tait

DOUBLE-diffusive convection is not to be mixed up with double-defective confusion. These different terms for the same phenomenon, the first employed by its proponents and the second by sceptics, may be taken as a light-hearted indication of a lively debate that has been taking place in the geological literature now for more than a decade. The debate concerns the types of convective effects that may occur in molten rock or magma, the stuff of volcanic eruptions, when it is resident in the Earth's crust in magma chambers that are part of subvolcanic plumbing systems. The protractedness of the debate arises not because geologists function as slowly as the processes that they study, but because of the fundamental difficulty of the scientific problem at stake and because it is hard to collect direct evidence to test ideas. On page 390 of this issue, Liang *et al.*<sup>1</sup> provide arguably the first convincing observations of double-diffusive convection taking place in melts which, although synthetic, have much in common with naturally occurring magmas.

Natural magmas show a wide range of chemical compositions and, as a result, of physical properties such as viscosity which determine their fluid dynamic behaviour and hence the frequency and type of volcanic eruptions. One of the central preoccupations of igneous petrology has therefore been to try to explain the chemical diversity of magmas. It has been accepted for some time that this variety cannot simply be the result of the melting of different regions of the interior of a heterogeneous Earth, as this would be inconsistent with what is known of the thermodynamics of magmas and would provide a poor and *ad hoc* explanation of field evidence. Indeed it can be plausibly argued that the principal igneous processes, partial melting and fractional crystallization, have a natural tendency to increase chemical diversity, and it is precisely because much of the Earth's mass has been through the mill of these processes over the aeons of geological time that we find ourselves living on a highly differentiated planet.

Single-component convection takes place in fluids in which one property (most commonly temperature) that can diffuse, and affects the density of a fluid, develops spatial gradients that are acted on by the force of gravity. Double-diffusive, or more generally multicomponent, convection can occur when two or more properties (typically temperature and chemical composition or the concentrations of several different chemical species) are present, if these diffuse at different rates

and have opposing effects on the density gradient in the fluid.

Two principal regimes are known that are quite different from single-component flows: layered convection and 'salt-finger' convection. Physical oceanographers are familiar with these from studying the ocean currents generated by gradients of temperature and salinity in the water. Probably the most counter-intuitive property of such flows is that convection can occur even when the overall fluid density decreases vertically upwards, when the gradient would appear to be stable. This happens because the different rates of diffusion of the various components present in the fluid can allow density anomalies to develop and so promote convection. The cooling and crystallization of magma in crustal reservoirs leads to spatial gradients of temperature and of its numerous chemical constituents, and it is now realized that multicomponent convective flows could play a key role in the chemical evolution of magmas. This subject is a nice example of cross-fertilization between scientific disciplines linked by a common physical principle<sup>2</sup>.

The additional twist that Liang *et al.* give to the story is their claim that not only are different components diffusing at different rates but also off-diagonal terms

in the multicomponent diffusion matrix play a crucial role in the redistribution of the species. That is to say, a diffusive flux of one component occurs because of spatial gradients of others. One can think of this phenomenologically as the chemical potential of a component being a function of its local environment because of weak bonding with other components: in short, non-ideality of the solution.

A note of caution may be wise here, as we do not know well what chemical species or complexes are really present in natural silicate melts. The compositions of silicate melts are conventionally expressed as proportions of metallic oxides, a convention followed by Liang *et al.*, but there is no guarantee that these are in fact the thermodynamically relevant components present and diffusing down chemical potential gradients in the melt<sup>3</sup>. A different convention will lead to different off-diagonal terms in the diffusion matrix. However, such off-diagonal terms are expected to arise in the case of a non-ideal solution — that is, when the activities of components are not proportional to their concentrations — and therefore although the numerical magnitudes of the off-diagonal terms will vary with the choice of components, even the most appropriate choice is unlikely to eradicate them.

The approach usually used in extending the ideas of multicomponent convection to geological problems has been to assume that the same fundamental phenomena that can be conveniently observed in

## Taking the beetle by the horns

THIS is the business end of the male dung beetle *Onthophagus acuminatus*, a native of Central America, which uses its formidable headgear in intraspecific battle. In some beetles in a population the horns are large, whereas in others they are absent or much reduced in size. Horn size is correlated with the size of the owner, but the trait is discontinuous; that is, male *O. acuminatus* can be divided into two distinct groups according to horn length.



D. Emlen

Is the explanation for this dimorphism to be found in hereditary or environmental factors? Douglas J. Emlen conducted a series of experiments to find which is the more likely (*Proc. R. Soc. B* **256**, 131–136; 1994). The particular environmental variable he altered was the amount of food — howler monkey dung — available to beetle larvae, and the answer was clear cut. Horn length is not primarily inherited but is a trait associated with body size and depends on nutrition. This observation, says Emlen, bears on two issues. First, it supports the idea that male secondary sexual characteristics evolve as signals of their possessors' quality. Second, it raises the question of what selective forces maintain the dimorphism. Some genetic 'switch' is probably involved early in a beetle's life, which means the question is as much one for developmental as evolutionary biologists.

T.L.



laboratory experiments with aqueous solutions will occur in any multicomponent liquid<sup>2</sup> — obvious differences in the scales and rates of processes notwithstanding. Extrapolations from tanks a few tens of centimetres in size, containing fluids at room temperature, to crustal reservoirs several kilometres in size containing magma at about 1,000 °C have been greeted with some scepticism. The work of Liang *et al.* may go some way to convincing sceptics that results obtained in experiments with aqueous solutions can be relevant to magmas. They cannot directly observe the convective flow because the melt is opaque, so instead they have used the elegant technique of quenching the experimental charge and subsequently analysing the distribution of elements in the sample, now transformed into a glass. They are thus able more or less instantaneously to freeze plumes at different stages of development and examine their form after opening up the experiment.

## GEOCHEMISTRY

## Earth's innermost secrets

François Guyot

THE formation of the Earth's core is one of the most remarkable events in the history of our planet, but little is known about the details of this process. So it was heartening to hear at a recent conference\* that advances in analytical geochemistry, and in high-temperature and high-pressure physics, are starting to give us a clearer picture of the first 100 million years of the Earth's history.

The simplest picture would be that the Earth was built up by steady accretion of homogeneous material from the solar nebula, and that this was followed by the differentiation of a metallic core. But most of the scientists who attended the meeting agreed that such models are unable to explain the global geochemical characteristics of our planet. Instead, it looks as though the abundances of various elements in the Earth's mantle are best explained by a first growth step under chemically reducing conditions, followed by accretion of more oxidized matter and then the eventual addition of a 'late veneer'. Such models have been aired for some years<sup>1-3</sup>, but the results of the workshop have considerably strengthened the case for inhomogeneous accretion.

Why is such a complex picture of the Earth's accretion necessary? Siderophile (iron-seeking) elements are strongly depleted in the mantle and crust of our planet relative to their mean abundances

The experiments of Liang *et al.* do not answer the broader questions of what the geometry of convection in magma chambers may actually be like and how fast chemical differentiation takes place. However, their results showing the first timid photographic appearance of a magmatic 'salt finger' caught in the convective act should stimulate new studies of multicomponent convection. Ultimately, these should complement laboratory results obtained with analogue fluids, such as aqueous solutions, by helping to test the scaling relationships used to extrapolate such results to the size of a natural magma chamber. □

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1. Liang, Y., Richter, F. M. & Watson, E. B. *Nature* **369**, 390–392 (1994).
2. Huppert, H. E. J. *Fluid Mech.* **212**, 209–240 (1990).
3. Kress, V. C. & Ghiorso, M. S. *Geochim. cosmochim. Acta* **57**, 4453–4466 (1993).

in the Solar System, as inferred from analyses of primitive meteorites. This is to be expected as a consequence of their partitioning into the metallic core. The problem is that they are still far more abundant in the silicate mantle than would be expected from the partitioning between iron and silicates measured in laboratory experiments. Even more surprising, the relative abundances of highly siderophile elements, such as gold, platinum, palladium, rhenium, osmium, ruthenium, rhodium and iridium, are very similar in the mean Solar System and in the Earth's mantle.

If there had been doubts about the accuracy of such results before, the new results presented at the meeting seem to have laid them to rest: many of the presentations emphasized advances in analytical geochemistry, and especially determinations of the composition of mantle rocks by inductively coupled plasma mass spectrometry, or by neutron activation analysis, which have confirmed the earlier suggestions (for example by Ted Ringwood<sup>4</sup>).

Clearly, such primitive relative abundances would be impossible if these elements had ever been fractionated between metal and silicates, because their partition coefficients are different. And although the moderately siderophile elements — such as iron, nickel, cobalt and tungsten — are not found in exactly primitive relative abundances in mantle rocks, nor are they in equilibrium with the

core. In the past, it had been suggested that the problem lay in extrapolating from laboratory results to core conditions, and that at high temperatures and high pressures, changes in the partition coefficients between iron and silicates could explain the observed abundance patterns. Now, though, numerous laboratory partitioning measurements spanning large ranges of temperature, pressure and oxygen fugacity have been made (many were shown at the meeting). These apparently rule out a single-stage separation of the core from the mantle, at least up to temperatures and pressures corresponding to the top of the lower mantle.

So we turn instead to models involving three accretion stages, which do seem to explain the geochemical data<sup>1-3</sup>. In the first stage, materials from the solar nebula are accreted under highly reducing conditions; dense, iron-rich metal separates from the silicates and percolates downwards to form the core, sweeping with it all the siderophile elements from the proto-mantle. In the next stage, oxidized primitive matter is added to the system and mostly remains in the mantle. A small fraction, however, migrates to the core, stripping the mantle of all the highly siderophile elements just introduced; the mantle abundances of the moderately siderophile elements are established at this stage. Eventually, a late-stage veneer establishes the abundances of the highly siderophile elements in the Earth's mantle.

To establish the details of this picture and to rule out single-stage homogeneous accretion models altogether, it will be necessary to extend the partitioning experiments up to ultra-high pressures (over 100 GPa) and ultra-high temperatures (over 4,000 K). Such experiments would be important, as physical models of core formation suggest a complex picture of exchange between metal and silicates under widely varying pressure and temperature conditions (D. J. Stevenson, California Institute of Technology). The definitive composition of the core is likely to be established by a subtle balance between mechanisms of metal migration within the mantle — the rapid fall of large metal masses, or the slower percolation of metal melt through solid silicates which would allow at least partial equilibration between the two phases (Stevenson).

Less of a consensus exists on the nature of the light 'impurity' element present in the core. This has been a longstanding question ever since geophysicists observed that the density of the outer core given by seismological models is substantially smaller than that of iron–nickel alloys measured experimentally under appropriate pressures and temperatures by shock-wave techniques. The difference is taken to imply the presence in the core of about 10 weight per cent of elements

\* European Association of Geochemistry Workshop on the Formation of the Earth's Core, Max-Planck-Institut für Chemie, Mainz, Germany, 18–20 April 1994.

whose atomic mass is lower than that of iron.

The wide agreement on an early chemically reducing stage has had the interesting effect of removing most objections against silicon as the light element in the Earth's core, since it is well known from metallurgical studies that silicon-bearing steels are made under reducing conditions. Moreover, silicon in the Earth's core is required if the upper mantle silicon abundance is representative of that in the whole mantle and if the bulk Earth has the composition of the most primitive meteorites (C. J. Allègre, Université de Paris).

As sulphur is not likely to constitute more than 3 wt% of the core (G. Dreibus, Max-Planck-Institut für Chemie, Mainz) and as oxygen dissolves in the metal only at very high pressures, the case for silicon as the major light element in the Earth's core has been strongly revived. Additionally, ultra-high-pressure experiments indicate that oxygen does not depress the melting point of the outer core liquid alloy (R. Boehler, Max-Planck-Institut für Chemie, Mainz), a condition generally required to crystallize the solid inner core at reasonable temperatures.

It must be stressed, however, that the nature and amount of the light element(s) in the Earth's core are still largely conjectural. Among the other candidates, carbon might be present at low abundances (less than 2 wt%) but nonetheless could be concentrated in the inner core as very stable iron carbide (B. J. Wood, University of Bristol). Another suggestion was that much of the Earth's hydrogen might be in the core (K. Kuramoto, University of Tokyo). It was even suggested (H. St C. O'Neill, Bayerisches Geoinstitut) that as heterogeneous accretion does not readily lend itself to the incorporation of large amounts of light elements (even including silicon) in the metal, the geophysical evidence for the amount of light impurities in the core should be re-examined.

Despite these uncertainties, the message to emerge from the meeting was cheering. It now seems possible to approach scientifically these erstwhile mythological issues with a combination of accurate geochemical observations, precise laboratory measurements and more realistic physical models. □

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# Ras blooms on sterile ground

Simon Cook and Frank McCormick

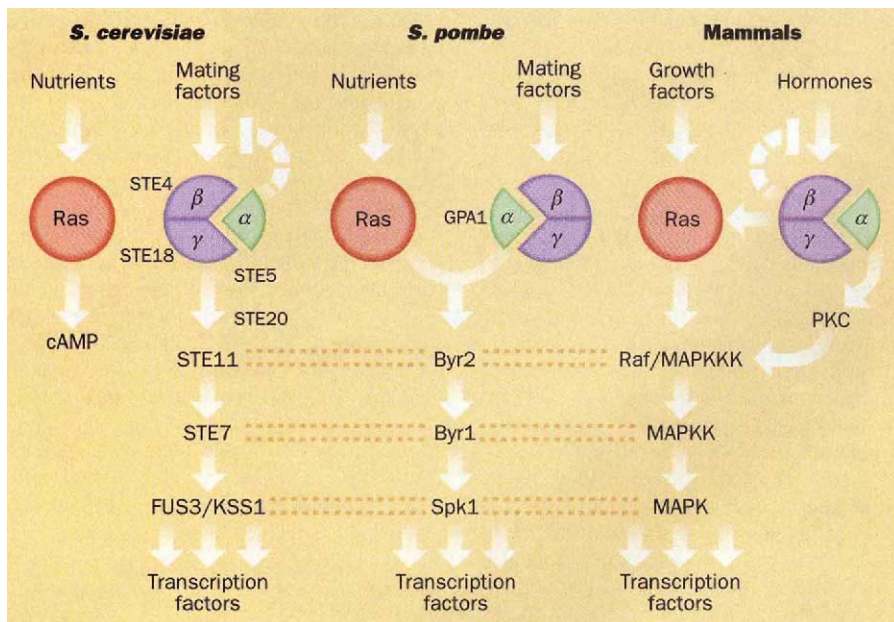
THE MAP kinase cascade, which transmits extracellular growth and differentiation signals into the cell nucleus, is highly conserved in eukaryotic organisms from yeast to man<sup>1</sup>. The protein components that activate the cascade in fly, worm and vertebrate cells (receptor tyrosine kinase, Grb2, Sos, Ras and so on) are also highly conserved, and their function is relatively well understood. But last year it turned out that, in mammalian cells, hormones that bind to G-protein-coupled or 'serpentine' receptors also require Ras to activate this kinase cascade<sup>2,3</sup>.

How then do these receptors couple to Ras? Do they activate tyrosine kinases which feed in at the level of adaptor proteins such as Grb2, or do they use another pathway? Faure *et al.* (in *Journal of Biological Chemistry*<sup>4</sup>) and Crespo *et al.* (page 418 of this issue<sup>5</sup>) take a major step by showing that  $\beta\gamma$  subunit dimers of G proteins can perform this function. This connection resembles aspects of the mating pheromone pathways of the budding yeast *Saccharomyces cerevisiae*<sup>6</sup>, hinting at the existence of another conserved pathway, one perhaps more ancient than that involving tyrosine kinases.

In *S. cerevisiae*, mating pheromones activate receptors that couple to a trimeric

GTPase consisting of  $\alpha$ -,  $\beta$ - and  $\gamma$ -subunits. The  $\beta\gamma$  dimer (encoded by *STE4* and *STE18* genes) is responsible for sending signals downstream to the MAP kinase cascade, participants in which are encoded by the *STE11*, *STE7*, *FUS3* and *KSS1* genes. The products of the *STE5* and *STE20* genes seem to lie between  $\beta\gamma$  and the *STE11* protein, but the precise epistatic relationship between these molecules remains to be defined<sup>6</sup>. In *S. cerevisiae*, the  $\alpha$ -subunit regulates the ability of  $\beta\gamma$  dimers to activate the kinase cascade and may be involved in the adaptive response to pheromone. The mating pheromone receptors of *Schizosaccharomyces pombe* (fission yeast) are also G-protein-coupled, but the analogous kinase cascade is activated by the G-protein  $\alpha$ -subunit (GPA1). In contrast to *S. cerevisiae*, Ras proteins are necessary for activation of the MAP kinase cascade in *S. pombe*<sup>6</sup>.

In mammalian cells, mitogens that use G-protein-coupled receptors (such as thrombin and lysophosphatidic acid) activate the MAP kinase cascade through Ras-dependent and independent pathways. However, it seems that Ras is necessary for sustained activation of MAP kinase and DNA synthesis by these mitogens<sup>3</sup>. It now appears that in mam-



The main components connecting G-protein-coupled receptors to the MAP kinase cascade in *S. cerevisiae*, *S. pombe* and mammals. Despite remarkable structural and functional conservation in the MAP kinase cascade itself (orange dotted lines), there are striking differences in the use of  $\alpha$ - and  $\beta\gamma$ -subunits as well as Ras to regulate this pathway. For example, in *S. cerevisiae*,  $\beta\gamma$  dimers activate the pathway independently of Ras while it now appears that mammals use both  $\alpha$ - and  $\beta\gamma$ /Ras-dependent pathways. In mammals Ras appears to be a common point of convergence for signals generated by receptor tyrosine kinases and G-protein-coupled receptors. Arrows represent pathways of signal propagation; thick dashed lines represent pathways of adaptive response to mating pheromones or hormones.

1. O'Neill, H. St C. *Geochim. cosmochim. Acta* **55**, 1159–1172 (1991).
2. Ringwood, A. E. *Proc. R. Soc. A* **395**, 1–46 (1984).
3. Wanke, H. *et al.* in *Archaeo Geochemistry* (eds Kröner, A. *et al.*) (Springer, 1984).
4. Ringwood, A. E. *Geochim. cosmochim. Acta* **30**, 41–104 (1966).



malian cells the MAP kinase cascade may be regulated by both  $\alpha$ -subunits and  $\beta\gamma$  dimers. Point mutations in  $\alpha_{12}$  which inhibit its GTPase (*gip2* mutant) cause oncogenic transformation of some cells and activate MAP kinase<sup>7</sup>.

The new results<sup>4,5</sup> show that the *gip2* protein and other  $\alpha$ -subunits give only weak activation of MAP kinase in COS cells. But transfection of  $\beta$  and  $\gamma$  together is sufficient to activate MAP kinase, analogous to the action of these proteins (Ste4p and Ste18p) when overexpressed in *S. cerevisiae*. Both groups also show that heterologous expression of the  $\alpha$ -subunit of transducin in order to sequester  $\beta\gamma$  dimers inhibits MAP kinase activation by co-transfected serpentine receptors (for example M1 or M2 carbachol receptors, D2 dopamine receptors and A1 adenosine receptors), but not by the endogenously expressed receptor for epidermal growth factor (EGF). So sequestration of free  $\beta\gamma$  dimers, liberated by receptor-mediated trimer dissociation, selectively blocks the signalling of serpentine receptors to MAP kinase.

The icing on the cake is provided by Crespo *et al.*<sup>5</sup>, who show that the ability of  $\beta\gamma$  to activate MAP kinase is inhibited by certain dominant-interfering Ras mutants; this suggests that  $\beta\gamma$  uses Ras to activate MAP kinase. In this respect, the mammalian system differs from *S. cerevisiae*. Finally, Faure *et al.*<sup>4</sup> address the issue of specificity by demonstrating that not all  $\beta\gamma$  dimers are active in this system. This suggests that only receptors linked to a particular  $\alpha\beta\gamma$  trimer combination may connect to Ras by this route.

How, then, are signals transmitted from  $\beta\gamma$  dimers to Ras? Studies on the interaction of  $\beta\gamma$  dimers with  $\beta$ -adrenergic receptor kinase<sup>8</sup> ( $\beta$ ARK, a negative regulator of the receptor involved in the adaptive response to hormone stimulation) provide some clues.  $\beta\gamma$  dimers bind to the pleckstrin-homology (PH) domain at the carboxy terminus of  $\beta$ ARK, thereby activating the kinase domain<sup>8</sup>. The  $\beta$ - and  $\gamma$ -subunits interact with each other through their amino termini and a model has been proposed in which the PH domain of  $\beta$ ARK,  $\beta$  and  $\gamma$  may form a tripartite coiled coil, resulting in activation and translocation of  $\beta$ ARK to the membrane<sup>9</sup>. The model remains speculative but provides us with a framework for considering how  $\beta\gamma$  may connect to Ras. Thus,  $\beta\gamma$  may bind to the PH domain of a Ras regulatory protein (the mammalian nucleotide-exchange factors, rasGRF and Sos, have PH domains, as does the GTPase-activating protein p120 GAP). Certainly  $\beta\gamma$  dimers can bind to a glutathione *S*-transferase fusion protein consisting of the PH domain of rasGRF *in vitro*, though with reduced affinity compared to the binding to  $\beta$ ARK-PH<sup>10</sup>. Reagents for testing these models are

readily available, so we can expect answers before too long.

Alternative models can be envisaged. For example, the pathway connecting activation of the lysophosphatidic acid receptor to Ras is sensitive to certain kinase inhibitors<sup>2</sup>. The equivalent pathway in *S. cerevisiae* involves a kinase (Ste20p); analogous proteins may therefore be involved in mammalian cells. A mammalian homologue of STE20 has been cloned, but it seems to be regulated by the Cdc42 family of Ras-related GTPases rather than by Ras itself<sup>11</sup>.

Comparison of yeast and mammalian systems, with respect to the mechanism by which G proteins activate the MAP kinase cascade, has been less illuminating than might have been hoped. In *S. cerevisiae*, one such cascade is activated by  $\beta\gamma$  in a Ras-independent fashion; in this organism Ras regulates adenyl cyclase in response to nutrient status. In *S. pombe* a related cascade is activated by  $\alpha$ -subunits and Ras, apparently converging on the pathway in a co-dependent manner. The new results suggest that mammalian cells may use  $\alpha$ -subunits in a Ras-independent pathway (perhaps involving protein kinase C) and  $\beta\gamma$  subunits in a Ras-dependent pathway.  $\beta\gamma$  is also involved in adaptive responses to hormones in mammalian cells, whereas in *S. cerevisiae* this function may be carried out by  $\alpha$ . It is certainly remarkable that such structurally conserved biochemical entities are used in different ways, given the conservation elsewhere in the pathway (see figure and ref. 1).

When the details of the pathways are better understood, unifying themes may emerge. One such theme may be the recruitment of signalling molecules to the plasma membrane. In mammalian cells, the EGF receptor recruits Grb2/Sos to the plasma membrane to activate Ras using SH2 and SH3 domains; Ras is in turn involved in translocation of Raf to the plasma membrane, where the kinase somehow gets turned on. By analogy,  $\beta\gamma$  may assemble a signalling complex at the membrane to activate Ras and other effector pathways by using PH domains to direct protein-protein interactions. □

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1. Egan, S. E. & Weinberg, R. A. *Nature* **365**, 781 (1993).
2. van Corven, E. J. *et al. Proc. natn. Acad. Sci. U.S.A.* **90**, 1257–1261 (1993).
3. Cook, S. J. *et al. EMBO J.* **12**, 3475–3485 (1993).
4. Faure, M., Voyno-Yasenetskaya, T. A. & Bourne, H. R. *J. biol. Chem.* **269**, 7851–7854 (1994).
5. Crespo, P., Xu, N., Simonds, W. F. & Gutkind, J. S. *Nature* **369**, 418–420 (1994).
6. Errede, B. & Lewin, D. E. *Curr. Opin. Cell Biol.* **5**, 254–260 (1993).
7. Gupta, S. K. *et al. J. biol. Chem.* **267**, 7987 (1992).
8. Inglesse, J. *et al. J. biol. Chem.* **268**, 23735 (1993).
9. Simonds, W. F., Manji, H. K. & Garritsen, A. *Trends biochem. Sci.* **18**, 315–317 (1993).
10. Touhara, K. *et al. J. biol. Chem.* **269**, 10217 (1994).
11. Manser, E. *et al. Nature* **367**, 40–46 (1994).

## Butcher, baker

BREAD, says Daedalus, is the most inspired of inventions. By a simple, even primitive process, a staple cereal grain is turned into food objects — loaves of bread — with a pleasing identity, taste and structure. They can be produced in endless and subtle variety, stored and traded, enhanced with other foodstuffs, and used as raw material in further cookery. Best of all, bread is inherently so satisfying that you can eat it every day of your life and never tire of it.

Another fundamental foodstuff is the sausage. It has the humbler goal of repackaging a wide range of animal scraps, fragments of offal and similar proteinaceous discards. Daedalus is now combining the philosophies behind these two great culinary inventions.

He is developing a very basic way of reclaiming reject protein material. DREADCO's biochemists are taking various food wastes, offal, old hides, insect and rodent carcasses, bacterial biomass and so on, and grinding them up in water. Heat, pressure and protease enzymes can then chop up their protein chains into lengths short enough to be soluble. The resulting peptides can be neatly filtered from the ghastly mixture as a sterile solution. Their chains are then enzymatically recombined into a sort of randomized 'average protein'. It approaches the thermodynamically most stable combination of the original amino acids. It is a gluey, pasty mass, showing no trace of its dubious origins.

As a foodstuff, average protein is pretty uninspiring. The problem is that it lacks structure. Daedalus is seeking a blowing reaction to expand average protein into a foamed solid, and a vulcanizing agent to stabilize the resulting bread-like product by cross-linking its linear chains into a three-dimensional molecular mesh. With luck, the yeast fermentation of added sugar will serve as a blowing reaction, as it does in baking bread. On later heating, the flavoursome Maillard protein-sugar reaction would then usefully develop the taste of the product. A dash of formaldehyde should harden and vulcanize it, though the result might resemble edible foamed Bakelite. A polyfunctional amino acid might do a better job.

The final product will be a splendid new mass-market foodstuff: a protein bread. It will be cheap, nutritious and satisfying, with a detailed texture and the sort of mild flavour that never palls, but enhances any foodstuff eaten with it. DREADCO's Marketing Department is already wondering what to call it. 'Meat Loaf' has the wrong connotations somehow.

David Jones

# Hobgoblin of phylogenetics?

**SIR** — The exchange between Stewart<sup>1,2</sup> and Sidow<sup>3</sup> on the use of parsimony versus model-based methods for phylogenetic inference highlights a surprisingly under-appreciated consideration in the choice of methods: the tradeoff between consistency<sup>4</sup> and efficiency (or power<sup>5</sup>). Although Sidow correctly notes that distance methods need not equate raw similarity with closeness of relationship, his argument for the superiority of model-based methods deserves closer scrutiny. Specifically, knowing that a method will correctly reconstruct the phylogeny given an infinite amount of data (= consistency) does not necessarily lead to preference for that method when only a finite amount of data are available. If the method is inefficient, requiring large amounts of data before converging on the true tree, attaching too much importance to consistency may be misguided.

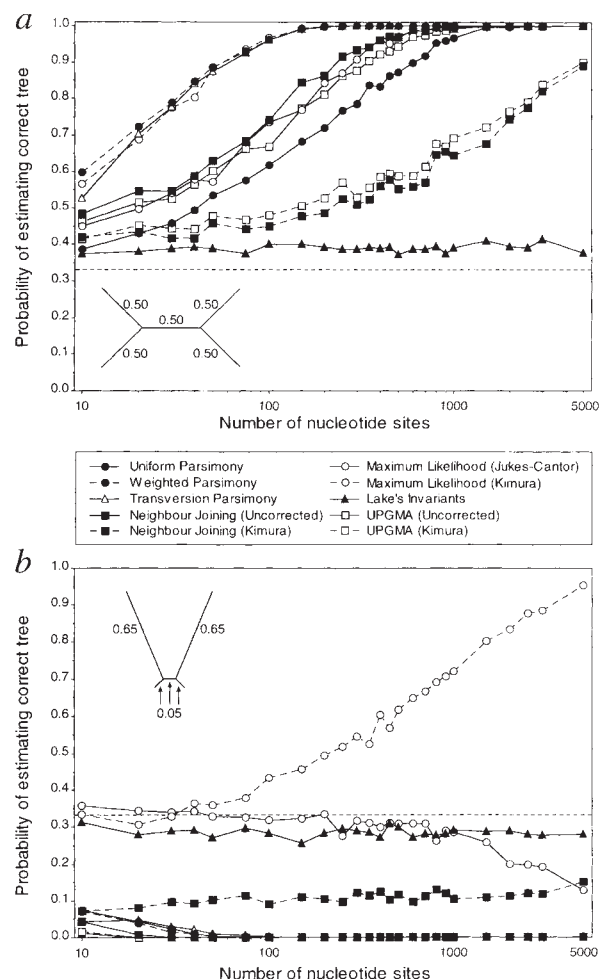
We illustrate this point with the computer simulation results shown in the figure. Most, if not all, phylogenetic methods make a consistent estimate of the phylogeny under the conditions of the first simulation (*a* in the figure), a Kimura 2-parameter model of evolution<sup>6</sup> with equal probabilities of change in all branches (and a relatively strong transition bias). However, many of these methods become inconsistent when certain branch-length inequalities are introduced (*b* in the figure). Unfortunately, the price of consistency throughout the parameter space of the Kimura model can be high. For the first simulation, the neighbour-joining method<sup>7</sup> using Kimura distances (corrected according to the same model used to generate the data) requires 5,000 nucleotides to reach the same level of accuracy in reconstructing the true evolutionary tree (90%) that standard parsimony achieves with only 600 nucleotides. Furthermore, parsimony analysis with transversions weighted more heavily than transitions reaches essentially 100% accuracy at only 300 nucleotides whether the transversion:transition weight is set to 2:1, 5:1, 10:1, or infinity (= transversion parsimony). Neighbour-joining with Kimura distances requires more than 50,000 nucleotides to achieve even 99% accuracy. Note also that the expected (average)

number of substitutions per site for the full tree is 5.2, exposing the common misconception that parsimony analysis "requires that each nucleotide has a negligible probability of having changed more than once" (ref. 3).

The simulation in *b* compares the performance of methods when some are known to be inconsistent. Neighbour-joining with uncorrected or Jukes-Cantor<sup>8</sup> distances, UPGMA, and all parsimony variants quickly converge on the wrong tree. Neighbour-joining with Kimura distances is consistent, but less effective than randomly choosing a tree (= 33% accuracy) for up to about 10,000 nucleotides. Lake's method of invariants<sup>9</sup>, although also consistent under the Kimura model, seems to have little to recommend it in this case; by the time its performance exceeds that of choosing a random tree (at about 100,000,000 nucleotides), neighbour-joining with Kimura distances and maximum likelihood (under the Kimura model) find the correct answer 50 and 100% of the time, respectively. Given the considerably poorer performance of neighbour-joining with Kimura distances under conditions where there is a reasonable chance of reconstructing the correct tree (simulation 1), we question the desirability of choosing it to achieve consistency under other conditions (simulation 2) for which it works barely half the time even when available sequence length exceeds the genome size of many organisms.

These simulations and many others we have performed (refs. 10–11 and our unpublished data) show the fallacy of Sidow's claim that "statistical methods" (lumping maximum likelihood with neighbour joining and invariant analysis) have all the "powers" of parsimony without its "pitfalls". Parsimony's performance is considerably enhanced when events that

have a higher frequency of occurrence (for example, transitions) are given less weight. Conditions do exist where parsimony will fail with certainty, but under these same conditions most "statistical methods" will also fail more often than not for sequences of even thousands of bases. Among statistical methods, maximum likelihood (which performed well in both simulations) is clearly preferable as a



Efficiency of various methods of phylogenetic inference for a four-taxon tree evolving under the Kimura model of evolution<sup>6</sup> with transitions ten times more likely than transversions and equal base frequencies. *a*, With all branches  $P = 0.5$  in length, where  $P$  is the probability that a difference will be observed in a site at the two ends of the branch (tree shown in lower left). *b*, With two opposing peripheral branches of  $P = 0.65$ , and  $P = 0.05$  for the remaining three branches (tree shown in upper left). All points are based on 1,000 simulated trees. Parsimony methods include transversion:transition weights of 1:1 (uniform parsimony), 10:1 (weighted parsimony), and infinity (transversion parsimony). The results for transversion:transition weights of 5:1 and 2:1 are not shown because of excessive overlap, but differ from results shown for 10:1 weighting by no more than 6% up to 200 nucleotides and are indistinguishable for longer sequences. Neighbour-joining (NJ) with Kimura distances requires 50,000 nucleotides (off scale shown) to achieve 99% accuracy under the conditions simulated in *a*. Lake's method of invariants is consistent under the conditions simulated in *a*, but has only a 94% chance of finding the correct tree at 100,000,000 nucleotide sites (off scale shown). The horizontal dashed lines indicate the probability of selecting a correct tree at random.

1. Stewart, C.-B. *Nature* **361**, 603–607 (1993).
2. Stewart, C.-B. *Nature* **367**, 26–27 (1994).
3. Sidow, A. *Nature* **367**, 26 (1994).
4. Felsenstein, J. *Syst. Zool.* **27**, 401–410 (1978).
5. Penny, D., Hendy, M. & Steel, M. A. *Trends ecol. Evol.* **7**, 73–79 (1992).
6. Kimura, M. *J. molec. Evol.* **16**, 111–120 (1980).
7. Saitou, N. & Nei, M. *J. molec. Evol.* **4**, 406–425 (1987).
8. Jukes, T. H. & Cantor, C. R. in *Mammalian Protein Metabolism* (ed. Munro, H. N.) 21–132 (Academic, New York, 1969).
9. Lake, J. A. *J. molec. Evol.* **4**, 167–191 (1987).
10. Huelsenbeck, J. P. & Hillis, D. M. *Syst. Zool.* **42**, 247–264 (1993).
11. Hillis, D. M. *et al. Science* (in the press).



criterion, but it is vastly more computationally intensive. Consequently, discovery of the optimal tree(s) is less likely due to the necessity of using more approximate tree searches. We agree with Sidow's desire for increased acceptance of model-based methods, but there are significant tradeoffs to be considered. The choice between these tradeoffs is, unfortunately, much more complicated than Sidow's "brief statistical guide".

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## Holocene mammoth dates

**SIR** — The reported persistence of dwarf mammoths well into the Holocene on Wrangel Island (S. Vartanyan, V. E. Garutt and A. V. Sher, *Nature* **362**, 337–340; 1993) has been called into question by some people who doubt the validity of the dates obtained in two Russian laboratories by conventional  $^{14}\text{C}$  dating. We have now submitted two previously dated dwarf teeth from Wrangel to the accelerator mass spectrometry (AMS) dating facility at the University of Arizona. Here are the uncalibrated results based on the 5568-year half-life from the paper by Vartanyan *et al.*:

| Sample | Conventional date and lab no. | AMS date and lab no.         |
|--------|-------------------------------|------------------------------|
| GUS-9  | 6,260 $\pm$ 5;<br>(LU-2799)   | 6,360 $\pm$ 60<br>(AA-11529) |
| PIK-1  | 7,250 $\pm$ 60<br>(LU-2809)   | 7,295 $\pm$ 95<br>(AA-11530) |

The extremely close correspondence of the dates corroborates both the veracity of the Russian results and the Holocene survival of the mammoths.

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## Mammoths in ancient Egypt?

**SIR** — Lister in News and Views<sup>1</sup> described new work<sup>2</sup> on the genus *Mammuthus* and its allies, suggesting that dwarf mammoths may have survived in northeast Siberia to coexist with the Egyptian pharaohs, and that dwarfed-mammoth populations and other dwarf

trunk is more like a reconstruction of a living mammoth than an immature elephant. The beast is probably unrelated to the modern mini-elephant reported from central Africa<sup>6,7</sup>. It looks different and no direct contact (transport of live animals) between that part of Africa and ancient Egypt has ever been documented. Both now and in the Pleistocene, miniature elephantids tended to segregate into miniature forms, which further suggests that the figure does depict a mature



elephantids survived on Mediterranean islands well into the Pleistocene. The figure, reproduced from a scene painted in a pharonic tomb<sup>3</sup>, is about the ivory trade, and raises the possibility that the elephantid represents a dwarf mammoth.

Egyptian artists could reproduce in colour two-dimensional identifying marks of living biological specimens very much like those in field-identifying manuals today. Thus specimens such as fish<sup>4</sup> and birds<sup>5</sup> can be placed into the modern frame of genus and species.

The figure represents tribute brought to Egypt and a parade of exotic animals. The bear is probably a sub-species of *Ursus arctos*, *U. arctos syriacus* or *U. arctos arctos*<sup>4</sup>. As native bears and modern man did not co-exist in Egypt<sup>4,5</sup>, the first bear seen would be as exotic to the Egyptians as the dwarf mammoth. This picture demonstrates the artist's ability to draw a creature alien to him. Similar bears existed in Asia (Palestine to Asia Minor), on some Mediterranean islands, south Europe and northwest Africa<sup>4</sup>.

The elephantid depicted here is not an immature elephant because of its large tusks. Its skull is domed, similar to a mammoth or possibly an Asian elephant. Its stance and the position of the tusks and

elephantid. Nevertheless, the man in the figure leading the animal is carrying two elephant tusks on his shoulder, and it is possible that the animal could be an elephant symbolic of the ivory's source rather than intended to be an accurate representation of its size.

If the elephantid, the tusks, the bear and the ingot (in the figure) came from the same source, one could speculate that they had a common origin.

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1. Lister, A. M. *Nature* **362**, 288–289 (1993).
2. Vartanyan, S. L. *et al.* *Nature* **362**, 337–340 (1993).
3. Bass, G. F. in *A History of Seafaring* (ed. Bass, G. F.) Ch. 1 p. 34 (Walker, New York, 1972).
4. Corbet, G. B. & Hill, J. E. *A World List of Mammalian Species* 2nd ed (Br. Mus. (nat. Hist.), London, 1986).
5. Osborn, D. J. & Helmy, I. *The Contemporary Land Mammals of Egypt* (Field Mus. of nat. Hist., Chicago, 1980).
6. Knapp, A. B. *World Archaeology* **24**, 332–347 (1993).
7. Sherratt, S. & Sherratt, A. *World Archaeology* **24**, 361–378 (1993).

### Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. Priority will be given to letters of fewer than 500 words and five references.

## Early diagnosis of Alzheimer's?

**SIR** — In the course of our immunohistological evaluation of a large number of olfactory mucosa collected at autopsy from patients of various ages, we noticed the appearance of abnormal  $\tau$ -protein in the olfactory nerve fibres even among young patients without dementia. Since the existence of  $\tau$ -positive nerve fibres in the olfactory mucosa can therefore not be interpreted as a change peculiar to Alzheimer's disease, it follows that, contrary to an earlier report<sup>1</sup>, biopsy of the olfactory mucosa cannot be used in the early diagnosis of Alzheimer's disease.

Olfactory abnormality<sup>2,3</sup> has been perceived as representing an early symptom of senile dementia. However, little has been done to correlate changes in the olfactory bulb<sup>4-7</sup> or in the olfactory mucosa<sup>1,8</sup>, the site of olfactory receptor neurons, with Alzheimer's disease. The usefulness of olfactory mucosal biopsy has been proposed for the early diagnosis of senile dementia<sup>1</sup>, and much attention has been focused on this proposal.

Alzheimer's disease is morphologically characterized by the frequent and intense appearance of the age-related changes that occur physiologically in senility. Therefore, histological and immunohistological examination of age-related changes in neuropsychiatrically non-demented subjects in sequence of age from youth to senility may contribute to the analysis of the early histopathology of Alzheimer's disease. We investigated the age-related changes using many olfactory mucosal tissues obtained at autopsy from patients (61 cases) of various ages and having had various diseases.

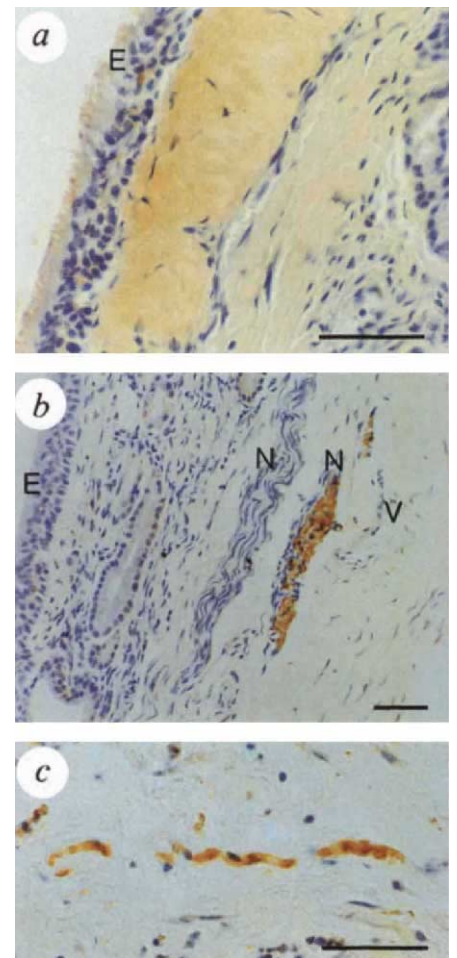
Underneath the olfactory epithelial layer, we observed a basal lamina-like structure that stained mildly eosinophilic with hematoxylin eosin stain but was not positive with periodic acid-Schiff stain. In

immunostaining with anti- $\beta$ -protein antibody<sup>9</sup> kindly provided by Drs Shin and Tateishi (Kyushu University), the basal lamina-like structure was recognized as a pale brown, pseudopositive-like band (*a* in the figure). This pseudopositive-like finding was obtained in 25.9% of the non-demented subjects. In the patients with Alzheimer's and Pick's disease examined in this study, we found no staining in the olfactory mucosa with anti- $\beta$ -protein antibody, although pseudopositive-like staining with anti- $\beta$ -protein antibody was observed more noticeably in subjects over 60 years of age. The implication of this finding remains unclear, but it seems to resemble the positive reaction of the epidermal/dermal junction to  $\beta$ -protein in Alzheimer's disease described by Joachim *et al.*<sup>10</sup>.

In immunostaining with an anti- $\tau$ -protein antibody<sup>9</sup> (also provided by Drs Shin and Tateishi), a positive reaction was obtained in 65.5% of the non-demented subjects. The youngest was a 19-year-old male (*b* in the figure). A positive reaction was also obtained in a 31-year-old male journalist who died of severe burns in a volcanic pyroclastic flow. In the non-demented subjects, the appearance of nerve fibres positive for anti- $\tau$ -protein antibody increased in frequency with age. In one of the two patients with Alzheimer's disease (a 91-year-old female), nerve fibres weakly positive for anti- $\tau$ -protein antibody were also observed. In most of the cases examined, anti- $\tau$ -protein antibody showed strong positivity in the sub-mucosal nerve-fibre bundles (*b*), although in some cases it stained positively in single fibres (*c* in the figure). Because axons from the olfactory epithelium exist as very thin fibres directly beneath the olfactory epithelial layer, it is usually difficult to detect them in a thin histological specimen. Even in the same section, nerve fibre bundles stained immunohistologically with anti- $\tau$ -protein antibody were coexistent with those not stained (*b*).

We did not observe any neurofibrillary tangles positive for anti- $\tau$ -protein antibody, such as those usually seen in the cytoplasm of hippocampal pyramidal neurons, in the cytoplasm of the olfactory epithelium in any of our cases including Alzheimer's and Pick's disease. Nor did we observe a senile plaque structure which could otherwise be recognized in the cerebral cortex from its strong positivity for anti- $\beta$ -protein antibody.

We have examined the hippocampus in 17 of the non-demented cases. We detected neurofibrillary tangles in the hippocampal pyramidal neurons in 11 (64.7%) of the 17 cases in which the olfactory mucosa showed positivity for anti- $\tau$ -protein antibody. Even in one of the patients with Alzheimer's disease who developed neurofibrillary changes and senile plaques in the hippocampus, the



Immunohistological characteristics of olfactory mucosa. *a*, Pseudopositive for anti- $\beta$ -protein antibody. A pale brown-stained band is seen at the position of the basal lamina-like structure. A 77-year-old male with diabetes mellitus. E, olfactory epithelium. *b*, Nerve bundles positive (right) and negative (left) for anti- $\tau$ -protein antibody. E, olfactory epithelium; N, nerve bundle; V, vessel. A 19-year-old male with sinusitis. *c*, Single nerve fibre positive for anti- $\tau$ -protein antibody. A 93-year-old male with myocardial infarction. Immunohistologically positive for  $\tau$ -protein. All scale bars, 0.05 mm.

olfactory mucosa reacted only feebly to anti- $\tau$ -protein antibody. Thus, the results of our morphological examinations produced no definite evidence to suggest a close relationship among the age-related changes seen in the two regions.

There have been strenuous efforts to establish an effective method for early diagnosis of senile dementia<sup>11-13</sup>. From the viewpoint of routine pathological diagnosis, highly unusual antibodies are not appropriate as tools for the early diagnosis of Alzheimer's disease: commercially available or easily procurable antibodies are preferable. For this reason, only the fewest possible and most easily available antibodies were used in this study. Notwithstanding the use of a limited range of antibodies, the existence of abnormal  $\tau$ -protein was confirmed in the olfactory

1. Talamo, B. R. *et al.* *Nature* **337**, 736-739 (1989).
2. Koss, E., Weiffenbach, J. M., Haxby, J. V. & Friedland, R. P. *Neurology* **38**, 1228-1232 (1988).
3. Rezek, D. L. *Archs. Neurol.* **44**, 1030-1032 (1987).
4. Kishikawa, M., Iseki, M., Nishimura, M., Sekine, I. & Fujii, H. *Acta Pathol. Jap.* **40**, 255-260 (1990).
5. Esiri, M. M. & Wilcock, G. K. J. *Neurol. neurosurg. Psychiat.* **47**, 56-60 (1984).
6. Ohm, T. G. & Braak, H. *Acta Neuropath.* **73**, 365-369 (1987).
7. Okamoto, K. *et al.* *Clin. Neurol.* **26**, 270-276 (1986) (in Japanese).
8. Trojanowski, J. Q., Newman, P. D., Hill, W. D. & Lee, V. M.-Y. *J. comp. Neurol.* **310**, 365-376 (1991).
9. Shin, R.-W., Ogomori, K., Kitamoto, T. & Tateishi, J. *Am. J. Path.* **134**, 1365-1371 (1989).
10. Joachim, C. L., Mori, H. & Selkoe, D. J. *Nature* **341**, 226-230 (1989).
11. Gunnerson, D. & Haley, B. *Proc. natn. Acad. Sci. U.S.A.* **89**, 11949-11953 (1992).
12. Matsubara, E. *et al.* *Ann. Neurol.* **28**, 561-567 (1990).
13. Jobst, K. A. *et al.* *Lancet* **340**, 1179-1183 (1992).
14. Lee, J. H., Goedert, M., Hill, W. D., Lee, V. M.-Y. & Trojanowski, J. Q. *Exp. Neurol.* **121**, 93-105 (1993).
15. Feng, W. H., Kauer, J. S. & Talamo, B. R. *Soc. Neurosci. Abstr.* **17**, 691 (1991).



mucosae even from relatively young subjects and subjects without any sign of dementia. This fact, we concluded, is sufficient to raise doubt about the validity of the method proposed in ref. 1.

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**TALAMO REPLIES** — Kishikawa *et al.* detect  $\tau$ -like immunoreactivity in nerve fibres in the sub-mucosal region (lamina propria) of the olfactory area of the nose in a various human autopsy cases, concluding that examination of nasal tissue would not be useful for the diagnosis of Alzheimer's disease. It is relevant to note that in our paper<sup>1</sup> we proposed that neuritic abnormalities within the epithelium, not changes in the deeper lamina propria, might be informative in the study of Alzheimer's.

Within the epithelium, neurofilament and  $\tau$ -like immunoreactivity are seen in these neurites, although neither is detectable in normal olfactory receptor neurons nor in other components of the sensory epithelium. We did not report finding prominent abnormal staining patterns with either of these antibodies in nerve bundles in the lamina propria, and did not suggest that  $\tau$ -immunoreactive fibres in the lamina propria were a feature peculiar to Alzheimer's.

Further, it is probable that the  $\tau$ -positive fibres described by Kishikawa *et al.* are not olfactory receptor neuron axons but are deep axon bundles of trigeminal or autonomic fibres expressing both  $\tau$  and neurofilament protein as normal constituents. Kishikawa *et al.* did not use antibodies (for example to olfactory marker protein) that would identify olfactory axon fascicles and clearly distinguish them from these other deeply located nerve bundles. In addition, the  $\tau$  antibody they used recognizes normal as well as abnormal  $\tau$ , so the observed immunoreactivity may not indicate neurodegenerative changes. This probably explains why Kishikawa *et al.* found staining in most normal individuals.

In agreement with Kishikawa *et al.*, we also did not find structures resembling neurofibrillary tangles, nor have we found evidence for plaques stained for  $\beta$ -amyloid peptide, when using antibody provided by D. Selkoe (ref. 1 and our unpublished results). The neurodegenera-

tive changes of olfactory epithelium thus seem to reflect those seen in degenerating brain and dystrophic neurites, but do not replicate them exactly.

A second issue raised by Kishikawa *et al.* is whether examination of the olfactory mucosa is likely to be useful diagnostically. In this case, the use of other probes must be considered. We find that neurodegenerative and phenotypic changes in human olfactory epithelium are not confined to Alzheimer's patients, although they are prominent in that group. Similar abnormalities are observed in a substantial number of non-demented patients (B.R.T. *et al.*, unpublished results; see ref. 8). A further extensive immunostaining study of the epithelial fibre masses with various  $\tau$  antibodies<sup>14</sup> suggested that one of the antibodies might preferentially react with particular epitopes in dystrophic olfactory neurites in patients with Alzheimer's. Although the olfactory changes do not appear to be associated exclusively with Alzheimer's, the cause for the transformation of the olfactory neurons is unclear; it remains possible that the events that trigger changes in brains affected by Alzheimer's also produce these neuritic changes in the

olfactory epithelium.

A question that is still open is whether nasal biopsy could be useful for studies of this type of pathology. Our early studies hinted that pathological regions of the epithelium might be dispersed irregularly among completely degenerated or relatively normal olfactory regions in autopsy tissue. We have now found<sup>15</sup> that the location of the pathological changes in the superficial epithelium is unpredictable from case to case, suggesting that random sampling by biopsy may not reveal the abnormalities. A possible solution to this difficulty would be to develop a method to identify visually the affected area using fibre-optic endoscopy.

In summary, the human olfactory pathology that we have observed appears to be primary epithelial and to involve olfactory neurons; we agree that staining of nerve bundles in the deeper nasal lamina propria with  $\tau$  antibodies does not appear to be informative for Alzheimer's disease or for other epithelial neuritic disorders.

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## No thermal instability in the Universe

**SIR** — We wish to correct the conclusion of C. H. (*Nature* **359**, 40–42; 1992), who proposed that if the Universe was reionized by decaying dark matter, the energy of the reionization could fuel an instability which would lead to structure formation. The mechanism proposed was that given a heat source uniformly distributed in space, in a lower density region each particle would receive more energy, increasing its relative temperature and thus thermal pressure. Decaying dark matter would provide a uniform source of fixed energy photons; if the photons are absorbed locally (if the medium is optically thick) and if they are absorbed only by hydrogen (so that each photon deposits the same amount of energy), it would also provide a uniform source of heating.

But this mechanism does not lead to an instability, because the lower pressure resulting from the lower density cancels the increased pressure from the higher temperature. That is, because the thermal energy added per volume is constant and the thermal pressure is a constant times the thermal energy density, a perturbation in density cannot lead (through this mechanism) to a perturbation in pressure. In the discussion leading to equation 2 of the paper cited above it was incorrectly assumed that  $Im(\delta \ln n)$  could be ignored; in fact (in this optically thick limit) it is of the same magnitude as the included terms and of opposite signs.

We have performed a complete linear analysis of reionization with a uniform ionizing source. The analysis includes radiative transfer, general expansion, ionization and photon number disequilibrium, thermal conduction, and Compton cooling. It does not include ionization of helium, collisional ionization, other mechanisms of cooling or spectral evolution. We used a plane-wave, harmonic expansion, valid for times and distances small compared to the size and age of the Universe. That analysis confirms that there is no heating instability, though for an extremely narrow region of parameter space it does allow for a cooling instability which is too weak to be cosmologically significant.

At the suggestion of Dr A. Meiksin, we have also searched for instabilities which can occur if the decay photons are sufficiently energetic to ionize helium (manuscript in preparation). Though this heating mechanism can be unstable, the instability is overwhelmed by the stabilizing influence of Compton cooling.

Although these results are not absolutely conclusive, they suggest that such instabilities are unlikely to be important in the formation of cosmic structure.

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# Emotional arguments

Stuart Sutherland

**The Muse in the Machine: Computers and Creative Thought.** By David Gelernter. Fourth Estate/Free Press: 1994. Pp. 211. £16.99, \$22.95.

MOST workers in artificial intelligence are intellectuals. In consequence, most of them believe that if they can write a program that reasons and solves problems as well as they do, they will have created a replica of the human mind; some even claim that the program will be conscious. In trying to construct programs in their own image they have, however, lost sight of one of the most important features of the mind, namely emotion. The great merit of *The Muse in the Machine* is that it tries to put emotion into artificial intelligence; its great weakness is that it fails.

David Gelernter argues that human thought is a spectrum, ranging from focusing on solving a particular problem ("directed thinking" in the words of the Gestalt psychologists), down to the inconsequential thinking characteristic of dreaming, day-dreaming, the thoughts of young children and (more doubtfully) those of the ancients. His main claim is that this unfocused thinking is driven by emotion: successive thoughts are related only by their emotional overtones, not by any concrete similarity between their objects. He argues that it is such thinking that underlies all creativity and that, above all, gives rise to useful analogies.

It is of course true that emotion colours thought: those who are depressed think of depressing events and are pessimistic about the future. But to argue that all creativity depends on emotional linkages seems a gross exaggeration. Gelernter cites the well known case of Kekulé solving the problem of the structure of benzene by imagining a ring of snakes. But it is hard to see an emotional link between snakes and the benzene molecule: the metaphor surely arises from a structural similarity, not an emotional one.

Gelernter makes the point that creative insights often occur when the mind is relaxed — Kekulé was dozing in front of his fire when the image of the snakes came to him. There is, however, a perfectly good existing account of why creative images often occur when the mind is idling: it has been known for 60 years that under intense concentration only the strongest associations occur. For weaker ones to emerge, including analogies that are not immediately obvious, attention has to be relaxed. Not only is this phenomenon well supported by evidence, there is a plausible theory (going back to the underestimated Clarke Hull) to explain it.

Gelernter maintains that emotions are

based on bodily sensations, presumably, although he does not say so, mainly those from the autonomic system. But although this system is implicated in states of high arousal, it is most unlikely that its activation could account for all the subtle differences in human emotions that he stresses. Stanley Schachter showed that the arousal produced by adrenalin was interpreted as happiness by people in a happy situation and as anxiety by those in an unpleasant one. Moreover, someone moved by a poem may feel the appropriate emotion with no arousal of the peripheral nervous system. Nor can facial expressions or bodily posture account for emotion — "One may smile and smile and be a villain".

Gelernter describes a computer program he wrote to simulate directed thinking. In essence it superimposes images of the same kind of scene viewed repeatedly. The representation of anything that occurs on every occasion will be strengthened so that when the scene is evoked again, those features will be the most salient and the easiest to recover. He claims that syllogisms can be solved in this way, but as the program, unlike associative networks, is not supplied with the concept of class inclusion, it is hard to see how this can be done.

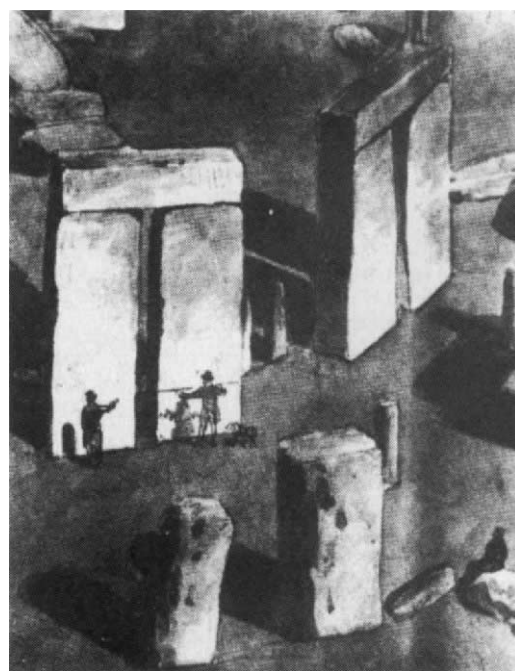
Oddly, and perhaps wisely, he has not attempted to write a program to simulate the role of emotion, although he believes this to be possible, by making the program move successively to representations that have the same emotional context but are in other ways completely different. He equivocates on whether such a computer would be conscious, eventually deciding that it would not be. He rightly observes that if someone says to a computer "I've lost my job" and the computer says "I understand", its reply is false, unless it can in some way feel the appropriate emotion. It may "understand" the facts, but not the blend of emotions engendered in the dismissed employee. It cannot empathize.

This point was made in

stronger terms by Joseph Weizenbaum many years ago. He pointed out that because of our biological nature we have drives such as hunger and sex, and the desire to love or be loved. We have these needs in order to survive and we are unhappy if they are unfulfilled. No computer could genuinely have them: at most it might exhibit a desire for the nearest electric plug. No matter how successful we were in simulating these emotions and drives on a computer, the program would still be a simulation, not the real thing. So there could be no reason to ascribe any form of consciousness to it.

Unfortunately Gelernter's writing and sometimes his thoughts tend to be rather jerky and he is given to annoying colloquialisms, such as "Nice try but no cigar" to describe an argument with which he disagrees. Moreover, he feels the need to support his own arguments by quoting banal remarks from such self-appointed sages as George Steiner ("Metaphor relates hitherto unrelated areas of experience") and Daniel Dennett ("Perception turns imperceptibly into memories"). Nevertheless his main thesis is aired too rarely and the book should be read by anyone interested in computers and the mind: it might even persuade some hackers to abandon their consoles from time to time and take a doze by the fireside. □

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MEASURING of Stonehenge early in the nineteenth century. Its design and proportions attracted the attention of architectural historians of the time. Taken from the revised and updated *Stonehenge Complete* by Christopher Chippendale. Thames and Hudson, £12.95 (pbk).



### Science Book Prizes

Steve Jones of University College London has won this year's £10,000 Rhône-Poulenc Science Book Prize for **The Language of the Genes: Biology, History and the Evolutionary Future** (Flamingo, £6.99 (pbk)/Doubleday in the United States; for a review see *Nature* **364**, 113; 1993). Out of 53 entries in the general category, six other books were shortlisted: **Afterglow of Creation** by Marcus Chown (Arrow, £5.99 (pbk); see *Nature* **366**, 373; 1994) and **The Red Queen: Sex and the Evolution of Human Nature** by Matt Ridley (Viking, £17.99; see *Nature* **367**, 697; 1994), both of which were highly commended; and **The Diversity of Life** by Edward O. Wilson (Penguin, £7.99 (pbk); see *Nature* **361**, 311; 1993); **Dreams of a Final Theory: The Search for the Fundamental Laws of Nature** by Steven Weinberg (Vintage, £6.99 (pbk); see *Nature* **361**, 697; 1993); **The Engineer in the Garden** by Colin Tudge (Cape, £17.99; see *Nature* **366**, 379; 1994); and **The Neandertals: Changing the Image of Mankind** by Erik Trinkhaus and Pat Shipman (Cape, £20; see *Nature* **363**, 591; 1993).

Three junior prizes were also awarded: £5,000 to Linda Gamlin for **Eyewitness Guide: Evolution** (Dorling Kindersley); £2,500 to Rebecca Heddle and Paul Shipton for **Science with Weather** (Usborne); and £2,500 to David Lambert for **The Ultimate Dinosaur Book** (Dorling Kindersley). The judges commented that many of the 32 junior entries lacked "flair and imagination" and often contained "inaccurate information", although the overall quality of books in the general category was "extremely high". The prizes are awarded for books judged to contribute most to the public understanding of science and technology. They are administered by the UK Committee for the Public Understanding of Science and by the Science Museum, and sponsored by the chemical and pharmaceutical company Rhône-Poulenc, which also funds a special print run enabling some 4,500 UK educational establishments for 16-year-olds and over to receive a free copy of the winning general book.

### New editions

**The Falling Sickness** by Owsei Temkin (2nd edn). Johns Hopkins University Press, £20.50 (pbk). A history of epilepsy from the time of Greeks to the beginnings of modern neurology, first published in 1945.

**Explorer of the Universe: A Biography of George Emery Hale** by Helen Wright. American Institute of Physics Press, \$34.95, £28. First published in 1966.

**Structure Determination by X-ray Crystallography** by M. F. C. Ladd and R. A. Palmer (3rd edn). Plenum, \$34.50.

## Ecology by definition

John Lawton

**The Concise Oxford Dictionary of Ecology.** By Michael Allaby. *Oxford University Press*: 1994. Pp. 415. £19.95, \$35 (hbk); £7.99, \$19.95 (pbk).

WHAT do nacreous clouds, hopeful monsters, flood forecasting and eigenvalues have in common? The answer is that they are among the 5,000 entries in this new dictionary. It is a measure of the breadth of topics ecologists need to study (and the



COLONY of bees taken from the well-illustrated *Animal Behaviour* edited by Tim Halliday. An international team of researchers covers topics on "Birth to Maturity", "Food and Shelter", "The Social Animal" and "Instinct and Intelligence". Blandford Press, £14.99.

difficulty of ensuring comprehensive coverage) that subjects include plant and animal physiology, behaviour, evolution and genetics, conservation, climatology and meteorology, geomorphology, oceanography, pollution, biogeography and taxonomy, as well as what most practitioners would regard as the core technical vocabulary.

By definition, a good dictionary must be both comprehensive and authoritative. How well does this one do? It is difficult to be certain without repeated use, by different people with a range of knowledge and interests, but this addition to the Concise Oxford stable seems authoritative. I found no definition with which I seriously disagree, and only a few with which I would quibble. Some idea of the breadth of coverage can be gained by picking a page at random. For example, on page 296

we find pergelic, periglacial, permanent quadrat, Permian, peroxyacetyl nitrates, pest and pH among the 29 entries. From this page alone, I discovered no fewer than eight new words (two of them listed here). Reading dictionaries can be fun.

Missing from this random sample, by chance, is an example of one of the bibliographic notes of "eminent ecologists and other scientists" that appear among the workaday words. Few living ecologists make it (E. P. Odum is an exception), but being dead and famous is no guarantee of inclusion. Elton is there and so is Fisher, but not Hutchinson, MacArthur, Lack or Nicholson, to name but four. My choice of individuals would have been rather different, or preferably longer. Nevertheless, this is a useful feature, particularly for students.

We live in a world not only of real words and people but also of acronyms. Here Allaby has an almost impossible task, and he might have been wiser not to try. USDA is defined, but not NSF or NERC; IBP but not IGBP. The reason is unclear. Much the same could be said about the few generic names included among the entries. Why, for example, select *Eichhornia* (the genus that includes water hyacinth) for special mention, and not other equally notorious weeds? The space saved could easily have been filled by more useful definitions.

Indeed, it is in the 'real words' that I found some worrying gaps and significant omissions. For instance, the dictionary deals more comprehensively with ecosystem ecology, pollution and environmental problems than with population biology and important new technologies. There is no mention of interference- or exploitation-competition, self-thinning, limit-cycles, regulation, guilds or indirect interactions. Geographic information systems, pixels and geostatistics are also conspicuous by their absence. In other words, it falls a little short of its dustjacket claim to be up to date, and it is clearly not as comprehensive as one might hope.

That said, the task of compiling such a dictionary is not an easy one. Ecology is a broad church, and it is probably impossible to please every member of the congregation. My guess is that this new, concise dictionary of ecology will please most users, most of the time. I shall certainly use it and so will my colleagues and students. That makes it valuable — by definition. □

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THIS photograph by Ronald Frommann of *Stern* magazine is one of about 190 prizewinning images brought together in *The World Press Photo Yearbook 1994* (Thames and Hudson, £9.95). His pictures of premature infants, some babies weighing as little as 500 grams, were taken at a centre in Dortmund, Germany, that specializes in high-risk births. The photos won second and third prizes in the Science and Technology section of the annual contest, which last year attracted more than 22,000 entries from photojournalists, agencies, newspapers and magazines around the world.

## Great minds and neuronal theories

Mitchell Glickstein

**Foundations of Neuroscience.** By Marcus Jacobson. Plenum: 1994. Pp. 387. \$79.50, £63.60.

NEUROSCIENCE is the study of the structure and function of the nervous system. Neuroscientists seek to learn how the brain and spinal cord process sensory signals and control movement, and ultimately to understand the role of the brain in its complex functions of language, thought and volition. Marcus Jacobson examines the foundations of neuroscience, its philosophical basis and its history. He asks what constitutes a theory in neuroscience and what sort of evidence led to the development of its theories. He finds it surprising that little has been written about these questions, and he disagrees with many of the approaches and conclusions of previous work. He argues that histories often give a false picture of the development of neuroscience as an orderly progression from ignorance to knowledge. Neuroscience, he demonstrates, has always had contradictions and controversies, and many more people have contributed to its development than a few honoured figures.

The author deals extensively with the neuron theory, which is the single most unifying theme of neuroscience. The theory says that the brain and spinal cord

are made up of individual elements called nerve cells or neurons, and that neurons are interconnected by contact; their processes do not fuse. Although the usual view is that the circuits formed by neurons can account for all phenomena that neuroscience seeks to understand, Jacobson hints that the exclusive focus on neurons may be mistaken. Glial cells greatly outnumber nerve cells, and perhaps they do more than just support and nourish them.

The author gives ample evidence that the neuron doctrine did not emerge fully blown from the mind of any one person. Rather, it evolved slowly over much of the nineteenth century, beginning with the pioneering work of Purkinjé and Schwann, and culminating with the contributions of Ramón y Cajal and Sherrington. As Jacobson points out, although Cajal and Sherrington strongly supported the doctrine, the evidence remained indirect. The gaps between neuronal processes are below the limits of resolution of the light microscope, so it was only with the greatly increased resolution of the electron microscope that there was final proof that the processes of nerve cells do not fuse.

Jacobson warns against excessive emphasis on the contributions of single individuals. Cajal, he argues, has been put on too high a pedestal. He draws a cartoon

of Cajal portrayed as a tiny man perched on the shoulders of a giant Camillo Golgi, another of the great nineteenth-century neurohistologists. Here I part company with Jacobson. Cajal stands head and shoulders above all other neurohistologists before and since. His great textbook on the histology of the nervous system, first written in Spanish about a hundred years ago, still serves as a guide for experimental research on the brain. Cajal's genius is clearest when his writing is compared with that of his contemporaries. None had so clear and lucid a style. None portrayed fundamental concepts of brain structures as deftly as Cajal could with a few diagrams. I remain an unreconstructed hero-worshipper.

Study of the history of neuroscience helps us to recognize both its progress and its limitations. Although advances may be hard to see from day to day, over a longer period the field makes orderly progress. It seems as if each generation of neuroscientists tries to account for the structure and function of the brain completely, with inconsistencies and lacunae papered over. Discoveries are often generalized prematurely. When the electrical activity of peripheral nerves began to be understood, there was an attempt to explain reflexes on the basis of principles derived from study of axonal conduction. Early discoveries of the receptive field properties of single cells in the visual cortex were quickly extrapolated to account for all forms of vision. In both cases the fundamental discoveries remained valid, but the initial enthusiasms became tempered with time.

Perhaps the weakest part of the book is the last chapter on ethics in neuroscience. Jacobson presents a list of rules for ethical behaviour in neuroscience — many of them can be assumed under the command "thou shalt not lie". Indeed, lying in science generally reverses the usual scale of morality. In ordinary life it is worse to steal from a friend than to lie to him. Lying in science is the greater crime, because it undermines the basic assumption of the scientific effort. But I knew that.

Jacobson has a broad grasp of the history and the philosophical implications of the field of neuroscience. Although his points are explicitly and clearly made, the book pre-supposes a good deal of knowledge about the field, so it may not be easy for non-neuroscientists to read. All the same, it does an important service by enquiring deeply into the origins and philosophical implications of the underlying ideas of neuroscience. □

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# Free for all

Jonathan Hobbs

**Sustainable Development for a Democratic South Africa.** Edited by Ken Cole. Earthscan/St Martin's Press: 1994. Pp. 247. £16.95 (pbk), \$45.

As South Africa makes its long-overdue transition from the fatally flawed ideology of separate development to addressing the needs of sustainable development, the creation of a political system that allows for the participation of all citizens in decision-making is crucial. That this is now happening so rapidly means that *Sustainable Development for a Democratic South Africa* is already out of date. But as a contribution to the continuing political process of development, it is valuable.

As with most conference proceedings, theoretical treatises are uncomfortably mixed with descriptive narrative, and some good papers lie alongside poorer ones. One suspects that the rush to have such a title on the bookshelves while South Africa is still front-page news is why some contributions are little more than badly re-formulated lecture notes. But a handful are both coherent and critical.

Ken Cole has brought together a diverse group of academics, mainly from the United Kingdom, and spiced them with a journalist, a trade unionist and an aid worker. They provide insights into expected reforms in agriculture, education, health, economics, employment, energy and so on. The emphasis on the socio-political dimensions of sustainable development heralds a new way of thinking in 'environmentalism', which for too long has been preoccupied with the white élite's worries about nature conservation. A South Africa led by the African National Congress (ANC) will inevitably concentrate on justice and full participation as pre-requisites of a healthy and productive environment. In other words, it will achieve a better balance between the priorities of rhinos and those of people, especially the poor; effective ecological protection will be achieved with a more equitable distribution of power.

The issues that the authors focus on as relevant to environmental protection include economic growth, the informal sector, nongovernment and community-based organizations, land reform, education, health and other social services. Some gaps exist. With a team of exclusively male authors it is not surprising that the role of women receives scant attention.

For the ANC successfully to transform itself from a liberation movement to a democratic government, the considerable economic potential of South Africa will have to be more effectively used to compensate the disadvantaged, otherwise dis-

illusionment will encourage conflict and violence. Colin Stoneman argues that the alleviation of poverty is feasible only with massive job creation. The neo-liberal economic orthodoxy — a dogma enshrined in the structural adjustment programmes of the World Bank and the International Monetary Fund — is interpreted as a potentially disastrous remedy for South Africa. Robert Fine, in a further attack on the free market, suggests that an interventionist, social-democratic regime is needed if the coercion and suppression of the disadvantaged, perceived to result from the neo-liberal policies of the World Bank, are to be avoided.

Agriculture is, of course, of upmost importance economically. Lionell Cliffe addresses the special requirements of sustainable agriculture, especially land reform, by drawing comparisons with Zimbabwe's inheritance of similar inequities from the then Rhodesia, where large-scale farms once lay next to semi-arid, overcrowded homelands engaged in subsistence agriculture.

South African business is not about to allow environmental sanctions to replace political and economic sanctions. Phil O'Keefe and John Kirby could have cited examples of trading partners that are already demanding evidence of the environmental credentials of South African businesses. Instead, in a disjointed chapter covering energy, minerals and wildlife management, they postulate that such demands might be made.

With South Africa no longer a pariah, opportunities for regional integration now exist. Barry Munslow's review of current events in Mozambique and Angola is particularly appropriate: people there have paid a heavy price for their support of the liberation struggles in Zimbabwe, Namibia and South Africa — heavier perhaps than South Africans themselves. Both countries are contradictions, being rich in resources yet classified as among the poorest nations in the world. In the recent words of a Maputo-based United Nations official: "nothing describes Mozambique better than the word potential — that's all I ever hear, potential, bloody potential".

Regrettably, Munslow's extrapolation of lessons learnt in these former Portuguese colonies is weak. If nothing else, he describes what could happen to South Africa if it falls into civil war. He concludes that South Africa owes these countries an immense moral debt, with their lands turned into 'killing fields' under South Africa's strategy of regional destabilization.

Historically, environmental policymaking in South Africa has provided little opportunity for public input and paid little recognition to individuals' rights. At best, participation has grudgingly been interpreted as a therapeutic exercise in one-

way information flow. The law can be an effective mechanism for public participation in environmental policy formulation, an issue handled well by François du Bois.

Improved provision of public services to all South Africans is also now a priority. After a discussion on primary health care, the implications of the growing AIDS epidemic are singled out for special attention. Cross-border migration, refugee influx, conflicts, droughts, militarization and the low status of women all serve to make South Africa a high-risk country.

In all of these issues, there is no doubt that a well-informed, educated, participating public will be important. But Benjamin Pogrand's chapter on press freedom, although interesting, fails to make any explicit reference to the main theme of sustainable development.

Arguably the best contribution is Charles Aubgre's review of nongovernment and community-based organizations. These organizations face a critical challenge — how to survive now that they have fulfilled their basic agenda, resistance to apartheid. Aubgre argues that some of them should channel their energies into sustainable development. A sophisticated nongovernmental network, probably unparalleled in Africa, has developed over decades of struggle against oppression; there is much to build on.

The book arose from a course held at the University of East Anglia in 1992 for 11 South African activists. One suspects that the lecturers learnt as much as the students. It is a shame that we are not treated to the first-hand experiences that must have been discussed.

The book does little to advance our understanding of sustainable development. Nor does it offer solutions to how the concept can be made operational (to its credit it is honest about the difficulties of this task). In the final chapter, Cole provides a good overview of the ideologies of sustainable development, but it would have been better if definitional problems were made clear at the start.

The final chapter ideally would summarize the diversity of interests in the issue, establish common themes and revisit the opportunities and constraints that exist in South Africa. Radical reform is implied throughout the book but few authors offer a practical interpretation of this goal. Under apartheid, the common enemy of the black majority was unambiguous; now the priorities are less clear.

Nonetheless, the book provides a useful introduction for students of sustainable development or technical tourists of the aid-and-development set, for whom South Africa is now a legitimate stop-over. □

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# Complete DNA sequence of yeast chromosome XI

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**The complete DNA sequence of the yeast *Saccharomyces cerevisiae* chromosome XI has been determined. In addition to a compact arrangement of potential protein coding sequences, the 666,448-base-pair sequence has revealed general chromosome patterns; in particular, alternating regional variations in average base composition correlate with variations in local gene density along the chromosome. Significant discrepancies with the previously published genetic map demonstrate the need for using independent physical mapping criteria.**

DESPITE recent technical developments<sup>1-4</sup>, the availability of complete sequences of complex genomes, such as those of mammals, is still remote. Thus, in an attempt to define functional genes more rapidly, particularly human ones, attention has turned to the sequencing of complementary DNA libraries<sup>5,6</sup>. In this situation, model organisms with small and compact genomes like bacteria<sup>7-9</sup>, or with genomes of intermediate sizes such as *Caenorhabditis elegans*<sup>10</sup> or *Arabidopsis thaliana*<sup>11</sup>, assume great importance as experimental subjects because their complete genomic sequences can be anticipated using current methodology. Of all model organisms, *Saccharomyces cerevisiae* occupies a unique position as it combines the advantages of being a eukaryote, being susceptible to powerful genetic techniques, and of having a genome size of only 13.5 megabases (Mb) (200 times smaller than that of the human genome). With yeast, the set of genes sufficient to build a simple eukaryotic cell can be deciphered with a relatively limited effort, and beyond its intrinsic biological significance can serve as a reference against which

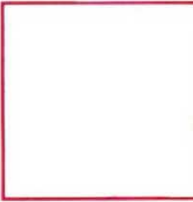
sequences of human, animal or plant genes may be compared.

Two years ago, a consortium of 35 European laboratories published the first complete sequence of a eukaryotic chromosome, that of chromosome III of *S. cerevisiae*<sup>12</sup>. The sequence revealed that almost half of the many new genes discovered had no homologue among previously described genes of either yeast or other organisms. But the experience with chromosome III was also important to help establish precise organizational rules for subsequent phases of the European Union genome-sequencing programmes<sup>13</sup>. During the past three years, our consortium has turned its efforts to the sequencing of yeast chromosomes II (820 kilobases (kb)) and XI. We report here the complete sequence of chromosome XI (666,448 base pairs (bp)), the second eukaryotic chromosome ever entirely sequenced. Beyond the many novel genes it contains, and the more precise description of the yeast genome composition it permits, the larger size of that chromosome, compared to chromosome III (315 kb), is sufficient to reveal new chromosomal organization patterns.

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**Chromosome XI map, deduced from complete sequence—see overleaf for details.**



## Assembly and verification of sequence

The sequence was determined from a set of 29 selected partially overlapping cosmid clones of a purpose-built genomic library (A.T. *et al.*, manuscript in preparation) from *S. cerevisiae* strain FY1679 (a direct derivative of S288C). Cosmids were distributed between the collaborating laboratories according to a scheme presented elsewhere (B.D. *et al.*, manuscript in preparation). Telomeres were physically mapped relative to the terminal-most cosmid inserts using I-SceI chromosome fragmentation and then sequenced from plasmids rescued from integrations into the terminal (C<sub>1</sub>-A)<sub>n</sub> repeats of chromosome XI (note that the number of such repeats retained in the present sequence is arbitrary). Sequencing strategies and methods were left to the initiative of each laboratory and were diverse. Sequences were considered as final and entered into the MIPS data library when all bases had been unambiguously determined on both strands and strategies had been examined by the DNA coordinator. Sequences were then compared to the detailed restriction map constructed for this work, further examined for the possible occurrence of frameshifts, and if necessary returned to the sequencing laboratories for additional verification. Independent verification was also used to confirm critical or difficult regions and to estimate the overall sequence accuracy (Table 1).

## Definition of ORFs and other elements

A total of 331 open reading frames (ORFs) were identified in the entire chromosome using principles explained in Fig. 1 legend. Seven of these ORFs are interrupted by introns. This list includes 43 partially overlapping ORFs (20 pairs and 1 triplet), all but four representing antiparallel overlaps. Six pairs each include a gene whose function is known, whereas nine other pairs and the triplet each include an ORF whose predicted product has a homologue in the databases, suggesting that it corresponds to a real gene (for details, see B.D. *et al.*, manuscript in prepara-

TABLE 1 Quality control and results of estimated overall sequence accuracy

| Method of verification                                                                | Total no. of fragments | Total bp verified | Error % detected |
|---------------------------------------------------------------------------------------|------------------------|-------------------|------------------|
| Original overlaps between cosmids                                                     | 28                     | 63,424            | 0.02             |
| Resequencing of selected segments (3–5 kb long)                                       | 21                     | 72,270            | 0.03             |
| Resequencing of random segments (~300 bp long)                                        | 71                     | 18,778            | 0.05             |
| Resequencing of suspected segments (~300 bp long) from designed oligonucleotide pairs | 60                     | 17,035            | 0.03             |
| Total                                                                                 | 180                    | 171,507           |                  |
| Overall sequence accuracy, 99.97%                                                     |                        |                   |                  |

All sequences submitted by collaborating laboratories to the MIPS data library were subjected to quality control. This included, initially, extensive re-examination of the restriction map, tracking down putative frameshifts, comparison with overlapping sequences from other laboratories and, eventually, the resequencing of selected or random segments on an anonymous basis. Selected segments were either 3–5-kb fragments that were entirely resequenced using the same methods, strategies and criteria as the original sequences, or short segments (~300 bp) chosen from suspected or difficult zones that were resequenced directly from cosmids using designated pairs of oligonucleotides as primers. Random segments were inserts of ~500 bp issued from shotgun cloning of the entire chromosome in the pBluescript SK + vector. The overall sequence accuracy has been estimated from extrapolation of the number of actual errors found in the original sequences after re-examination of each divergence with the verification sequences.

tion). In all such cases, the partially overlapping partner ORF is shorter, suggesting that it may not correspond to a real gene. The reality of ORFs as functional genes has been systematically examined using, as criteria, their codon adaptation index (CAI; ref. 14) in conjunction with their size (we have verified that there is no general correlation between CAI and ORF size; data not shown). Although there exist functionally defined genes with CAI < 0.110, the average CAI of the subset of 93 chromosome XI genes with known functions (Fig. 1, and see below) is 0.211 ( $\sigma = 0.148$ , range 0.101–0.868), significantly higher than that of the entire set of 331 ORFs (average 0.170;  $\sigma = 0.112$ , range 0.045–0.868). To test the possibility that some predicted ORFs may occur by chance, a random sequence of the same size and composition as chromosome XI (both mononucleotide and dinucleotide frequencies are respected) has been generated. This sequence shows 37 ORFs longer than 100 codons (all are shorter than 150 codons; average is 115, with  $\sigma = 11.7$ ). Most of them have a low CAI (average 0.107;  $\sigma = 0.023$ , range 0.053–0.168). For this reason, ORFs that are both shorter than 150 codons and have CAI < 0.110 are considered as 'questionable' (Fig. 1). Consistent with this set containing many unreal genes, 11 out of the 23 questionable ORFs are partially overlapping, whereas only four of them have homologues (compared to 67% for all ORFs). The reality of ORFs as functional genes may also be challenged by the possible existence of pseudogenes. Although very few are known in the yeast genome, this might be the case for YKR103w and YKR104w, which are in the same frame but separated by a single stop codon (verified by sequencing yeast genomic DNA itself) and whose expected products are homologous to the N- and C-terminal parts of the same protein, respectively. From these considerations, we estimate the total number of chromosome XI ORFs corresponding to real genes to be ~310. To this number could be added a few ORFs, not shown in Fig. 1, that are shorter than 100 codons but have a very high CAI (B.D. *et al.*, manuscript in preparation).

A total of 16 transfer RNA genes, three of them containing short introns, and eleven  $\delta$  and one  $\sigma$  sequences have also been

FIG. 1 The map on pages 372–373 represents chromosome XI of *S. cerevisiae* as deduced from the complete sequence. The map is drawn to scale from the sequence (coordinates are in kb). The two DNA strands are materialized as colour gradient bars to represent compositional variations (Fig. 3). The top strand (designated 'Watson' strand) is oriented 5' to 3' from left to right. The sequence has been interpreted using the following principles: (1) all intron splice-site/branch-point pairs were listed; (2) all ORFs containing at least 100 contiguous sense codons (including the first ATG) and not entirely contained within a longer ORF on either DNA strand were listed (this includes partially overlapping ORFs); (3) the two lists were merged and all splice-site/branch-point pairs occurring inside an ORF but in opposite orientation were disregarded. The possible occurrence of reading frames flanking putative splice sites was then examined for all remaining pairs (namely those occurring inside ORFs in direct orientation or in inter-ORF regions). In this case, no lower size limit applied, but the existence (after splicing) of an ATG codon in-frame with the stop codon was essential. Finally, tRNA gene (red boxes), centromere, telomeres (grey boxes),  $\delta$ ,  $\sigma$  and  $\tau$  elements (green boxes) were sought by comparison with a previously characterized dataset of such elements. (H. Feldmann, unpublished results). This procedure identified 331 ORFs (blue boxes), which have been numbered in increasing order from the centromere and designated YKL for the left arm and YKR for the right arm (w/c suffix indicates the Watson/Crick coding strand). Twenty-two ORFs shorter than 150 codons and having a CAI < 0.110 were considered as questionable (light-blue boxes). The same has been applied to YKL118w (CAI 0.136) as it overlaps a  $\delta$  sequence. Predicted ORF translation products have been compared to sequence data libraries using the FastA, and BlastX algorithms as well as protein pattern search regimes: 93 ORFs correspond to genes whose functions have been identified (brown boxes above the chromosome map); 93 other ORFs have homologues of known function (for details, see B.D. *et al.*, manuscript in preparation). Parts of this sequence have been published<sup>17,29–52</sup> during the course of this programme (note that there are some differences as the published sequences had not been submitted to final quality controls of the entire chromosome).

identified (Fig. 1). All  $\delta$  sequences occur less than 300 nucleotides upstream of a tRNA gene (or less than 300 nucleotides upstream of the first  $\delta$  in cases of double  $\delta$ s). The  $\sigma$  sequence partially overlaps the 5' end of the tRNA Lys1 gene. These elements represent the long terminal repeats of yeast retrotransposons (Ty) but, in contrast to chromosome III (ref. 15), no complete Ty was found.

### Analysis of predicted protein products

Comparison of the present sequence with public databases revealed that 93 of the 331 ORFs (28%) correspond either to previously known protein-encoding genes or to genes whose functions have been determined during this work (Fig. 1 and B.D. *et al.*, manuscript in preparation). All other ORFs, 72% of the total, represent novel putative yeast genes whose functions need to be experimentally determined. But 93 of them (another

28% of the total) have homologues among gene products from yeast or other organisms whose functions are known, whereas 37 others (11% of the total) have homologues that are themselves of unknown function. The remaining 108 ORFs (33% of the total) either have no homologues in data libraries or show levels of similarity of uncertain significance (note that this last set includes 18 questionable ORFs). Overall, about 40–44% of the genes of chromosome XI are thus of unpredicted function, a figure similar to that of chromosome III (ref. 16).

### Organization of the chromosome

The very high gene density previously found with chromosome III is confirmed: ORFs occupy on average 72% of the sequence of chromosome XI. The average ORF size is 488 codons (1,464 bp): the longest ORF is YKR054c (4,092 codons), which encodes dynein<sup>17,18</sup>, and the second longest is YKL203c (2,473 codons), which is the *TOR2* gene<sup>19</sup>. Only 25 other ORFs are more than 1,000 codons long. The mean sizes of inter-ORF distances are 804 bp for 'divergent promoters' and only 381 bp for 'convergent terminators', with 'promoter-terminator combinations' being of intermediate length (orientation of ORFs, and hence that of transcription units, is statistically random and approximately equal numbers occur on each DNA strand). There are only six inter-ORF regions that are longer than 3,000 bp; five of these are located close to the telomeres, the sixth is found half-way along the left arm and contains a tRNA gene.

The size of chromosome XI (~5% of the total yeast genome) is sufficient to examine general features of chromosome organization which, combined with previous chromosome III data, should be informative about the yeast genome in general. The average base composition is 38.1% G + C (38.5% for chromosome III), with significant variations between ORFs (average of 40.2%), 'divergent promoters' (36.3%) and 'convergent terminators' (29.8%). Again, 'promoter-terminator' combinations are of intermediate value. The corresponding value for introns is 32.3%. Average base composition is symmetrical over the entire chromosome (the symmetry being even more apparent with dinucleotide frequencies; Fig. 2a), but this only reflects the almost equal numbers of ORFs encoded on each DNA strand, the base composition of ORFs themselves showing a significant excess of homopurine pairs on the coding strand (Fig. 2b). A regional variation of base composition was noted along chromosome III, with a major (G + C)-rich peak in each arm<sup>20,21</sup>. Variations of similar amplitudes are found here but, owing to the larger size of chromosome XI, a much more orderly pattern appears, with an almost regular periodicity of the G + C content (Fig. 3). Four major (G + C)-rich peaks occur on the left arm, separated from each other by ~90–100 kb, and one major peak (plus a minor one) occurs on the right arm (which is shorter). Re-examination of chromosome III indicates that spacing is similar. Thus a yeast chromosome appears as a succession of (G + C)-rich and (G + C)-poor segments of ~50 kb each (see Fig. 1 for an overall view of the phenomenon). Interestingly, the compositional periodicity correlates with local gene density (Fig. 4), as is the case in more complex genomes in which isochores of composition are, however, much larger<sup>22</sup>.

The present sequence also reveals interchromosomal subtelomeric duplications. For example, two left subtelomeric segments of the present sequence (coordinates 348–1,005 and 1,666–2,850) are almost identical with two segments of the right subtelomeric region of chromosome III. Conversely, the right subtelomeric part of the present sequence (from 658 kb to the end) matches almost perfectly the left subtelomeric region of chromosome III over more than 8 kb, the only exception being that a Ty5-1 element is inserted into chromosome III (ref. 23). Such duplications include three ORFs on the left arm and two ORFs on the right arm (Fig. 1). Subtelomeric regions of chromosome XI do not contain Y' elements (although, intriguingly, the 36-bp repeat internal to Y' ORF2 sequences<sup>24</sup> is found in a short

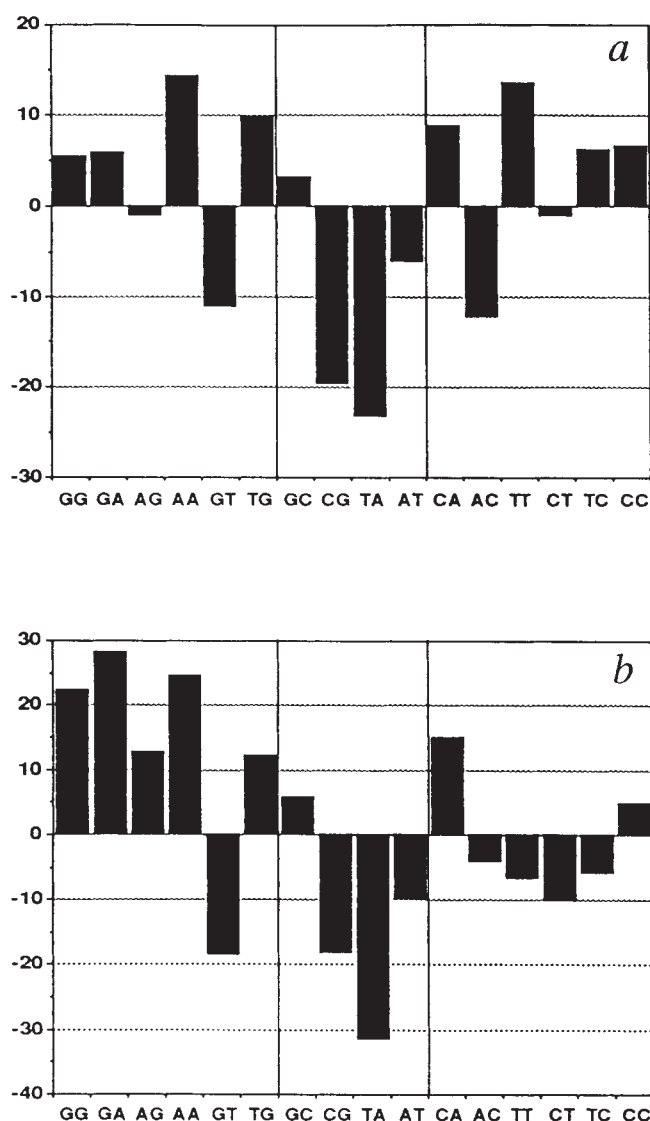


FIG. 2 Compositional symmetry/asymmetry of the chromosome and of its constitutive elements. Relative deviations of dinucleotide frequencies ((observed minus expected)/expected) are shown as vertical bars (expected frequencies are calculated from mononucleotide frequencies). Complementary dinucleotide pairs have been arranged in mirror image to help visualize compositional symmetry or asymmetry. Self-complementary dinucleotides are at the centre. a, Data for the entire chromosome, calculated from the Watson strand; b, data for ORFs only, calculated from the coding strand.



ORF located in the middle of the left arm; B.D. *et al.*, manuscript in preparation).

### Physical and genetic maps compared

The last edition of the genetic map of *S. cerevisiae*<sup>25</sup> assigned 50 genes or markers to chromosome XI, of which 43 were positioned on a single linear array and seven remained unmapped. Comparison of this map with that deduced from the complete sequence is shown in Fig. 4, which reveals major discrepancies in the gene order. The entire *URA1-STE6* segment, located next to the left telomere (positions ~25–46 kb of the present sequence), was translocated and inverted in the genetic map. *URA6*, which lies in the left arm, was erroneously mapped to the right arm, and the entire centromeric region, including the

3 centromere-linked genes *MET14*, *VPS1* and *PAP1*, was inverted. *GAP1*, which lies between *DAL80* and *TRK2*, was mapped distal to *TIF1*. Finally, a small inversion can also be noted between the two closely linked genes *CDC16* and *MAK11*.

### Discussion

Our 'network' approach to systematic genome sequencing started with chromosome III (refs 12, 13, 26) and has been improved here by the construction, before sequencing, of a high-quality cosmid library and a fine-resolution physical map of the chromosome and by the implementation of novel quality controls. The important discrepancies between the physical and genetic maps were a surprise. The physical map has been constructed without any reference to the genetic map from a complete set of overlapping cosmid clones derived from a unique strain, which have been individually verified for their colinearity with genomic yeast DNA. It is entirely consistent with direct physical measurements of the yeast chromosome after chromosome fragmentation using *I-SceI* (ref. 27) and was eventually confirmed by the final sequence. The genetic map, on the contrary, is a compromise between results obtained by several laboratories working independently and on different strains. It is possible that some strains, used to establish particular linkages, showed inversions or translocations with respect to the strain used for sequencing. But, in any case, this experience with yeast, where genetics are precise and easy, demonstrates the need for independent physical and genetic mapping data for all genome projects.

The rationale behind our effort on quality control was that if systematic genome sequencing of yeast is to be useful and significant, then sequence accuracy must permit correct interpretations. With the gene density and ORF size distribution of yeast, even relatively rare sequencing errors (many of which are missing nucleotides) result in a large fraction of the protein-coding genes being affected by frameshifts. A simple calculation predicts that with an accuracy of 99%, virtually all predicted genes contain errors, and that at 99.9% accuracy (a figure commonly obtained with good sequencing practices and regarded as satisfactory), two-thirds of the genes still contain at least one error (most often a frameshift). A higher level of accuracy (99.97%) has been achieved in this project only after costly effort; this figure is unlikely to be bettered with current technology, but still implies that about one-third of predicted genes will contain sequencing errors that will affect their interpretation.

In this work, the present sequence has been determined in its entirety, irrespective of the existence of previous sequence data, making *a posteriori* comparisons meaningful. From 107 different entries, totalling 288,252 bp, which correspond to parts of the present sequence but were determined independently by others (EMBL data library, release 73), we found only 26 (24%) of them to be identical to the present sequence. The others (76%) show divergences that range from 0.01% to >4%; the average being 0.3%, or 10 times the error-rate estimated for this study (we have excluded from our calculation those errors found at the extremities of published sequences which correspond to vector contaminations or weaker gel readings). Divergences with published sequences are of several types, but only some of them can plausibly be explained by strain differences. In the cases of the partial sequences published independently by the authors of the present work (a total of 23 entries totalling 309,467 bp; see Fig. 1 legend for refs), only 6 base substitutions and 13 additions/deletions exist compared to the present sequence, consistent with our estimated error rate of 0.03% (remember that these sequences had not yet been submitted to independent verification when published).

Much is still to be learned from large-scale sequencing programmes regarding genome organization and evolution, but the almost periodic variation of base composition and gene density found for chromosome XI is a strong indication that there exist rules in a eukaryotic chromosome that individual genes must

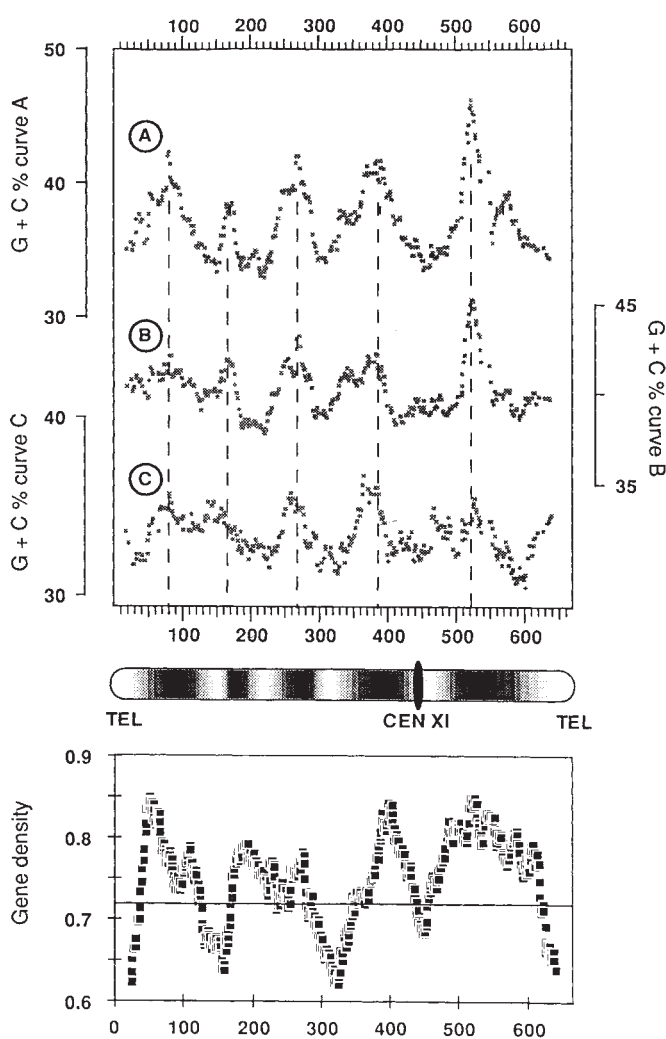


FIG. 3 Compositional variation and gene density distribution along chromosome XI. Top, Compositional variation along chromosome XI calculated as in ref. 20. Each point represents the average G + C composition of 15 consecutive elements but similar results were obtained for averages of 10–30 elements (not shown). Curve A: G + C composition calculated from the silent positions of codons only; curve B: G + C composition calculated from entire ORFs; curve C: G + C composition calculated from inter-ORF regions. Centre, Regional compositional variation along chromosome XI is shown as graduated shading: (white, G + C-poor; dark, G + C-rich). Bottom, Gene density along chromosome XI. Gene density is expressed as the fraction of nucleotides within ORFs versus the total number of nucleotides in sliding windows of 50 kb (steps are 1 kb). Similar results were obtained for sliding windows of 30–70 kb (not shown). Horizontal line represents average gene density for the entire chromosome (0.72).

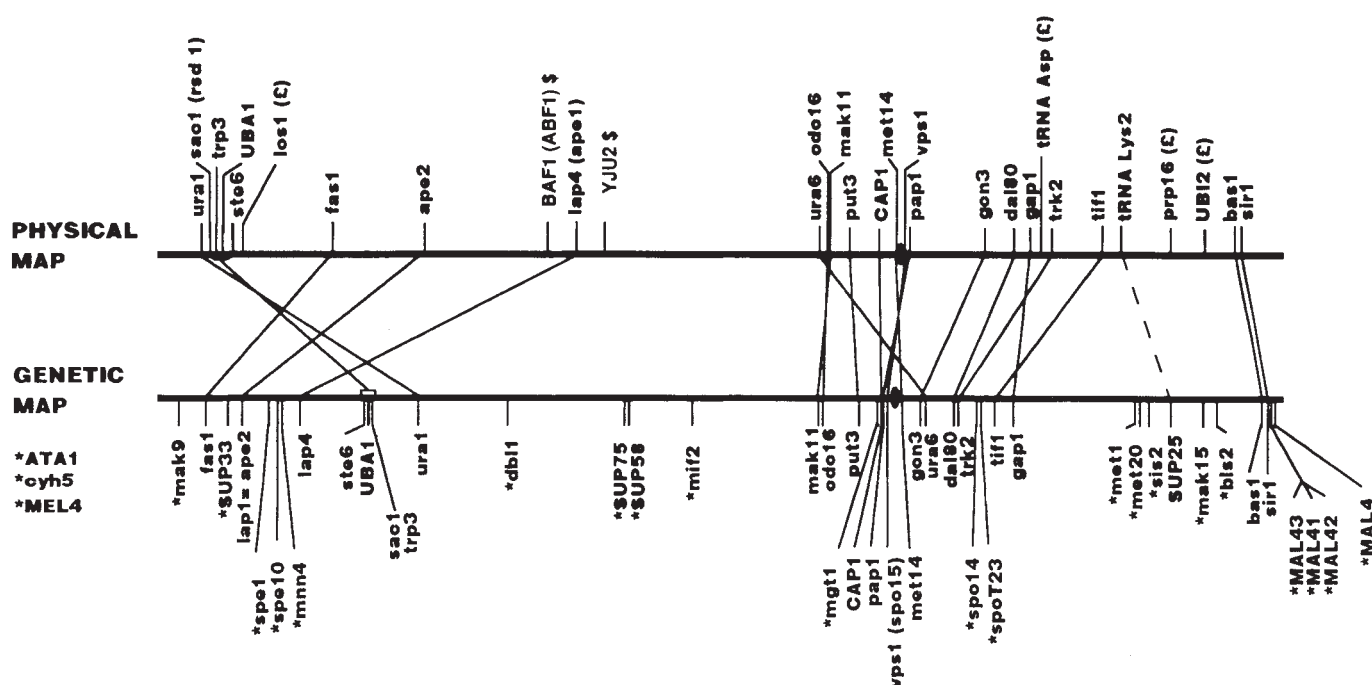


FIG. 4 Comparison of the genetic and physical maps of chromosome XI. The genetic map is redrawn from ref. 25. The physical map deduced from this work has been drawn to the same scale. Filled oval represents the centromere. The genetic map assigned 50 genes or markers to chromosome XI, of which 43 were positioned on a single linear array and 7 remained unmapped. Twenty-four of the 43 mapped genes and 4 of the 7 unmapped genes (*LOS1*, *PRP16*, *UB12* and *tRNA Asp*) could be unambiguously assigned to an ORF or a tRNA gene of the present

sequence on the basis of previous partial sequence data, use of probes or gene function (the remaining 22 genes or markers are indicated by an asterisk). The two genes *BAF1* (*ABF1*) and *YJU2* indicated by a dollar sign have previously been erroneously mapped to chromosomes V and X, respectively, on the basis of imprecise electrophoretic karyotype analysis<sup>53,54</sup>. Many other genes described here were not previously assigned to a chromosome (compare Figs 2 and 4). The assignment of *tRNA Lys2* to *SUP25* is only tentative.

obey, and which are location-dependent. Such rules may influence the expression of genes, their stability towards mutagenic forces or their recombination properties. The reason for the fairly regular succession of (G+C)-rich and (G+C)-poor segments along yeast chromosomes is unclear. A correlation with the location of replication origins or their order of firing during S phase might be imagined. However, the map of functional ARS elements for chromosome III (ref. 28) does not easily fit the distribution of its compositional peaks (functional ARS elements have yet to be defined for chromosome XI). In addition, many ARS are dispensable. Alternatively, the compositional periodicity of a yeast chromosome could reflect the folding of that chromosome, its attachment to the nuclear matrix or structural elements involved in chromosome segregation, or in the 'homology search' that precedes synapsis in the early meiotic prophase.

Insight into eukaryotic genome organization and evolution can also be gained from the degree of internal genetic redundancy. Determination of this value is, however, very imprecise at this stage of the yeast sequencing programme. If we extrapolate to the entire yeast genome, the fact that 15 of the chromosome XI ORFs of unknown function have homologues among ORFs also of unknown function and lying on other systematically sequenced chromosomes or on chromosome XI itself, we obtain the surprising figure that genetic redundancy in the yeast genome must be high, at least among the genes of unknown function.

The availability of the complete sequences of yeast chromosomes III and XI offered a chance to search for interesting or unexpected genes. Opinions may vary as to what constitutes an interesting gene, but among those are certainly the homologues to genes that perform differentiated functions in multicellular organisms (such as the *Drosophila* white-pigment gene on chromosome III; ref. 12), or are involved in human pathologies such as xeroderma pigmentosum (YKL113c) or adrenoleukodystrophy (YKL188c) on chromosome XI. The existence of such genes

in yeast, where their role remains to be clarified, may offer a powerful experimental system to identify their function or to develop new therapeutic agents. About a quarter of all predicted ORF products of chromosome XI have homologues in cDNA databases (including the human expressed sequence tags sequences), enabling genes common to all eukaryotes to be directly identified. Interestingly, this fraction is much higher (42%) among the 186 ORFs of known or predictable functions and much lower (<7%) among the remaining 145 ORFs of unknown functions. It looks as if many of these novel functions only required transient or low-level transcription in complex organisms or were primarily phylum-specific. Such questions may quickly be answered as systematic sequencing of the yeast genome progresses (chromosomes I, II and VI are nearly completed and all others are being sequenced at present), and new routes are explored to interpret the wealth of information. □

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- Smith, L. M. et al. *Nature* **321**, 674–679 (1986).
- Ansorge, W., Sproat, B. S., Stegemann, J. & Schwager, C. J. *Biochem. biophys. Meth.* **13**, 315–323 (1986).
- Pohl, F. M. & Beck, S. *Meth. Enzym.* **155**, 250–259 (1987).
- Church, G. M. & Kieffer-Higgins, S. *Science* **240**, 185–188 (1988).
- McCombie, W. R. et al. *Nature Genet.* **1**, 124–131 (1992).
- Okubo, K. et al. *Nature Genet.* **2**, 173–179 (1992).
- Daniels, D. L., Plunkett, G., Burland, V. & Blattner, F. R. *Science* **257**, 771–778 (1992).
- Kunst, F. & Devine, K. *Res. Microbiol.* **142**, 905–912 (1991).
- Honore, N. et al. *Molec. Microbiol.* **7**, 207–214 (1993).
- Wilson, R. et al. *Nature* **368**, 32–38 (1994).
- Meyerowitz, E. M. & Pruitt, R. E. *Science* **229**, 1214–1218 (1985).
- Oliver, S. G. et al. *Nature* **357**, 38–46 (1992).
- Vassarotti, A., Dujon, B., Feldmann, H., Mewes, H. W. & Goffeau, A. *J. Biotech.* (in the press).
- Sharp, P. M. & Li, W. H. *Nucleic Acids Res.* **15**, 1281–1295 (1987).
- Wickstead, B. L. et al. *Yeast* **10**, 39–57 (1994).
- Koonin, E. V., Bork, P. & Sander, C. *EMBO J.* **13**, 493–503 (1994).
- Eshel, D. et al. *Proc. natn. Acad. Sci. U.S.A.* **90**, 11172–11176 (1993).
- Li, Y. Y., Yeh, E., Haus, T. & Bloom, K. *Proc. natn. Acad. Sci. U.S.A.* **90**, 10096–10100 (1993).
- Kunz, J. et al. *Cell* **73**, 585–596 (1993).
- Sharp, P. & Lloyd, A. *Nucleic Acids Res.* **21**, 179–183 (1993).
- Karlín, S. et al. *Nucleic Acids Res.* **21**, 703–711 (1993).



22. Bernardi, G. *Gene* **135**, 57–66 (1993).
23. Voytas, D. F. & Boeke, J. D. *Nature* **358**, 717 (1992).
24. Louis, E. J. & Haber, J. E. *Genetics* **133**, 559–574 (1992).
25. Mortimer, R. K., Contopoulou, R. & King, J. S. *Yeast* **8**, 817–902 (1992).
26. Vassarotti, A. & Goffeau, A. *Trends Biotech.* **10**, 15–18 (1992).
27. Thierry, A. & Dujon, B. *Nucleic Acids Res.* **20**, 5625–5631 (1992).
28. Dershowitz, A. & Newlon, C. S. *Molec. cell. Biol.* **13**, 391–398 (1993).
29. Alexandraki, D. & Tzermia, M. *Yeast* (in the press).
30. Bossier, P., Fernandes, P., Villela, L. & Rodrigues-Pousada, C. *Yeast* (in the press).
31. Bou, G. *et al.* *Yeast* **9**, 1349–1354 (1993).
32. Boyer, J., Pascolo, S., Richard, G.-F. & Dujon, B. *Yeast* **9**, 279–287 (1993).
33. Chéret, G. C. *et al.* *Yeast* **9**, 1259–1265 (1993).
34. Colleaux, L., Richard, G. F., Thierry, A. & Dujon, B. *Yeast* **8**, 325–336 (1992).
35. Düsterhöft, A. & Philippsen, P. *Yeast* **8**, 749–759 (1992).
36. Garcia-Cantalejo, J. *et al.* *Yeast* **10**, 221–245 (1994).
37. Jacquier, A., Legrain, P. & Dujon, B. *Yeast* **8**, 121–132 (1992).
38. James, C. M., Gent, M. E. & Oliver, S. G. *Yeast* **10**, 257–264 (1994).
39. James, C. M., Gent, M. E., Indge, K. J. & Oliver, S. G. *Yeast* **10**, 247–255 (1994).
40. Pallier, C. *et al.* *Yeast* **9**, 1149–1155 (1993).
41. Pascolo, S. *et al.* *Yeast* **8**, 987–995 (1992).
42. Purnelle, B., Skala, J., Van Dyck, L. & Goffeau, A. *Yeast* **8**, 977–986 (1992).
43. Purnelle, B., Skala, J., Van Dyck, L. & Goffeau, A. *Yeast* **10**, 125–130 (1994).
44. Purnelle, B. *et al.* *Yeast* **9**, 1379–1384 (1993).
45. Rasmussen, S. W. *Yeast* (in the press).
46. Rasmussen, S. W. *Yeast* (in the press).
47. Singer-Krüger, B. *et al.* *J. Cell Biol.* (in the press).
48. Tzermia, M., Horaitis, O. & Alexandraki, D. *Yeast* (in the press).
49. van Vliet-Reedijk, J. C. & Planta, R. J. *Yeast* **9**, 1139–1147 (1993).

50. Vandenbol, M. *et al.* *Yeast* (in the press).
51. Vandenbol, M. *et al.* *Yeast* (in the press).
52. Wiemann, S. *et al.* *Yeast* **9**, 1343–1348 (1993).
53. Rhode, P. R., Sweder, K. S., Oegema, K. F. & Campbell, J. *Genes Dev.* **3**, 1926–1939 (1989).
54. Forrova, H., Kolarov, J., Ghislain, M. & Goffeau, A. *Yeast* **8**, 419–422 (1992).

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## LETTERS TO NATURE

## Is the ring around SN1987A a protostellar disk?

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ACCORDING to conventional wisdom<sup>1–4</sup>, the ring around supernova 1987A is a product of winds from the progenitor star, which should have produced a thin, dense, spherical shell<sup>1</sup>. It was accordingly a surprise when images obtained by the Hubble Space Telescope<sup>5–8</sup> revealed that the gas is in fact disposed in a thin ring, with a radial velocity<sup>9</sup> much smaller than that predicted by theory. This could be explained by an asymmetry in the red giant wind<sup>10–12</sup>, or by rotational flattening<sup>13,14</sup>, but these explanations seem to us to be *ad hoc*, and have associated problems. Here we propose, instead, that the ring is the inner rim of a disk of gas that is left over from the time of formation of the progenitor star. The centre of the disk was evaporated by the ionizing radiation of the progenitor over its lifetime of about ten million years, leaving a ring-like structure. Our hypothesis naturally explains the ring's physical properties, and leads to the prediction that we should see the rest of the disk shortly after the supernova ejecta hit the ring in AD 1999 ± 3.

During its red giant stage, the progenitor of SN1987A should have expelled relatively dense gas in a low velocity ( $v_R \approx 20 \text{ km s}^{-1}$ ) stellar wind. Then, several thousand years ago, the progenitor became a blue giant and the high-pressure bubble due to its fast ( $v_B \approx 10^3 \text{ km s}^{-1}$ ) stellar wind began to pack the inner part of the relic red giant wind into a thin, dense spherical shell<sup>1</sup>. Therefore, when evidence for circumstellar gas near SN1987A appeared in the form of narrow ultraviolet and optical emission lines<sup>2,3</sup>, it was natural to assume that the line-emitting gas must have been expelled by the supernova progenitor and was most likely the spherical shell resulting from the interacting winds. The overabundance of nitrogen inferred from the ultraviolet emission-line strengths was persuasive evidence in favour of the ejection hypothesis<sup>2,4</sup>. Other observations pose problems for this simple picture, however. In addition to the images<sup>5–8</sup>

showing that the gas is in a thin ellipse (apparently the projection of a circular ring inclined at  $\sim 45^\circ$ ), the radial velocity of the ring inferred from the velocity gradient along the minor axis<sup>9</sup> is  $\sim 10 \text{ km s}^{-1}$ , much less than the expansion velocity  $\sim 50 \text{ km s}^{-1}$  predicted by the interacting winds model.

In an attempt to explain why the circumstellar gas resides in a ring, several authors<sup>10–12</sup> proposed a modified model in which the red giant progenitor ejected its envelope in a wind that was much denser at the equator than at the poles. Then, when the progenitor became a blue giant, the pressure due to the blue giant wind would have moulded the inner boundary of the red giant wind into an hourglass-shaped shell. According to this interpretation, the ring is the waist of the hourglass and the outer bipolar nebulosity consists of those parts of the lobes of the hourglass that have been illuminated by the ionizing flash of the supernova.

There are reasons to be dissatisfied with this model, however. Numerical simulations<sup>12</sup> show that large flux anisotropy in the red giant wind is needed to produce a significantly flattened structure. But even with an equator/pole density ratio  $\sim (5\text{--}20)/1$ , the resulting ring shape appears to be thicker than observed (thickness/radius ratio  $\approx 0.1$ ). A thinner ring is possible, but requires even greater anisotropy in the red giant wind. There is no dynamical account for such a high degree of anisotropy. Furthermore, because the red giant wind is assumed to be accelerated by the blue giant wind, the expansion velocity should be greater than that of the red giant wind. Yet the observed radial velocity ( $10 \text{ km s}^{-1}$ ) is significantly less than the expansion velocity ( $\sim 15\text{--}20 \text{ km s}^{-1}$ ) of most red supergiant winds<sup>15</sup>. To account for the low radial velocity of the ring, the numerical simulations require a red giant wind with an extremely high equatorial mass loss rate and a very low ( $\sim 5 \text{ km s}^{-1}$ ) radial velocity.

It has been suggested<sup>13,14</sup> that the small aspect ratio of the ring may be caused by rotational flattening if the ejection is accelerated by a hypothetical companion<sup>16</sup>. If the red giant wind has a velocity of  $\sim 10 \text{ km s}^{-1}$ , only companions with binary separation comparable to, or smaller than, a few astronomical units, can provide sufficient acceleration. But in this case, the maximum specific angular momentum (per unit mass) that can be attained by the wind would fall short of the value required for rotational flattening at  $\sim 1$  light yr by two orders of magnitude.

Indeed, we are so uncomfortable with the ejection hypothesis that we think it worthwhile to advance an entirely different

model for the origin of the ring: namely, that it is the inner rim of a relic protostellar disk<sup>17</sup>. As the reader will see, the disk hypothesis can account for the ring naturally without invoking any *ad hoc* mechanisms, and it can be verified in a few years when the supernova envelope strikes the ring.

Suppose, then, that the observed ring is the inner rim of a keplerian gas disk that has been ionized by the initial ionizing flash of the supernova. (In a keplerian disk, the gas flows approximately in circular orbits according to Kepler's third law.) We know that the ring has radius  $R \approx 6.1 \times 10^{17}$  cm. If the supernova progenitor has mass  $M_* = 20 M_{20} M_\odot$  (where  $M_\odot$  is the solar mass), the Kepler velocity of the ring is  $v_K \approx 0.66 M_{20}^{-1/2} \text{ km s}^{-1}$  (too low to observe spectroscopically), and the orbital period is  $t_K \approx 1.8 \times 10^6 M_{20}^{-1/2} \text{ yr}$ .

We note that massive stars are generally observed to be rapid rotators<sup>18</sup> and probably formed from rotating clouds. During the collapse of rotating clouds, the fluid element with most of the angular momentum arrives in the outer regions of the disk where the specific angular momentum is expected to be larger than its average value<sup>19</sup>. In the present case, the specific angular momentum of this rotationally supported ring is  $\sim 4 \times 10^{22} \text{ cm}^2 \text{ s}^{-1}$ ; this value is an order of magnitude larger than the average specific angular momentum of the ring around  $\beta$  Pictoris<sup>20</sup> and of rotating molecular cloud cores<sup>21</sup> but comparable to some rapidly rotating cloud cores<sup>22</sup>. It has been proposed<sup>23</sup> that such relic disks may account for the "bow-tie" or "butterfly" geometry of some planetary nebulae<sup>24</sup>.

From the brightness and rate of fading of the ultraviolet lines<sup>2,4</sup>, we know that the emitting gas in the ring has mass  $M_r \approx 0.04 M_\odot$  and atomic density  $n_r \approx 2.5 \times 10^4 \text{ cm}^{-3}$ . (There must also be a component of lower density gas, with density  $n_l \approx 3 \times 10^3 \text{ cm}^{-3}$  in order to account for the [O III] 5,007 Å emission seen at late times.) Moreover, we may estimate that the ring has half thickness  $z \approx 6 \times 10^{16}$  cm. An upper bound on  $z$  is provided by the angular resolution of the Hubble Space Telescope image<sup>7</sup>, and a lower bound comes from the requirement that the ring must subtend a solid angle great enough to capture  $\sim 10\%$  of the ionizing photons from the initial flash of the supernova<sup>25</sup>, in order to account for the strength of the ultraviolet lines. In fact, the mass of the ring inferred from the narrow emission lines is probably determined by the net number of ionizing photons from the initial flash.

The self-gravity of the disk will exceed the vertical component of gravity due to the progenitor if the disk has density  $\rho \geq M_*/(4\pi R^3)$  or atomic density  $n \geq 10^4 \text{ cm}^{-3}$ . (Equivalently, the gravitational stability parameter  $Q \equiv c_s \Omega / \pi G \Sigma < 1$ , where  $c_s = (kT/1.3m_H)^{1/2} = 0.25 \text{ km s}^{-1} T_{10}^{1/2}$  is the isothermal sound speed,  $T_{10} = T/(10 \text{ K})$ ,  $m_H$  is the hydrogen atomic mass,  $\Omega$  is the angular velocity,  $G$  is Newton's constant, and  $\Sigma$  is the surface density of the disk.) Thus, we see that the disk is marginally self-gravitating. We can estimate its vertical scale height by equating self gravity to thermal pressure:  $z \approx c_s / (4\pi G \rho + \Omega^2)^{1/2} \approx 2 \times 10^{17} \text{ cm}$  ( $T_{10}/n_4)^{1/2} (1+Q)^{-1/2}$ , where  $n_4 = n_r/(10^4 \text{ cm}^{-3})$ ). Thus, if the cold gas in the disk has temperature  $T = 10 \text{ K}$  and density  $n_4 = 2.5$ , it should have half-thickness  $z \approx 10^{17} \text{ cm}$ , as observed. If the gravity of the central star is included, the thickness may decrease by a factor of  $\sim 2$ .

We estimate approximately the radius,  $R_i$ , to which photoionization by the progenitor removes the inner part of the disk as follows. Assume that the progenitor produces ionizing photons at a rate  $S_{47} = S_i/(10^{47} \text{ s}^{-1}) \approx 1$  (about right for a  $20 M_\odot$  star<sup>26</sup>) during its main sequence lifetime,  $t_{MS} \approx 10^7 \text{ yr}$ . If every incident ionizing photon removed one atom from the disk, the photo-erosion front would advance with velocity  $\dot{R}_0 = S_i/(4\pi R^2 n_r) \approx 10 \text{ km s}^{-1}$  for  $n_r = 2.5 \times 10^4 \text{ cm}^{-3}$ . But that assumption would not be correct because the recombination timescale,  $t_{rec} \approx (n_r \alpha_B)^{-1} \approx 3 \text{ yr}$ , where  $\alpha_B$  is the recombination rate coefficient, is much less than the timescale  $t_{flow} \approx z/c_s \approx 2,000 \text{ yr}$  for gas ( $T \approx 10^4 \text{ K}$ ) to flow away from the disk at sound speed  $c_s \approx 10 \text{ km s}^{-1}$ . Because only a fraction  $f_i \approx t_{rec}/t_{flow} \approx 1.5 \times 10^{-3}$

of the photoionizations actually results in an ion being removed from the disk, the photo-erosion front should advance with velocity  $\dot{R}_i \approx f_i \dot{R}_0 \approx 10^{-2} \text{ km s}^{-1}$ . Thus,  $R_i \approx \dot{R}_i t_{MS} \approx 5 \times 10^{17} \text{ cm}$ , fortuitously close to the observed radius of the ring.

The disk may also be pushed outward by the momentum flux carried by the incident radiation. A marginally self-gravitating disk should have mass  $M_d \approx 2\pi z R^2 n_r 1.3 m_H \approx (z/R) M_* \approx 2 M_\odot$ . For a gas column density  $\rho z \approx 3 \times 10^{-2} \text{ g cm}^{-2}$  along its mid-plane, the disk would be opaque to micrometre-sized grains. But grains may collide and coagulate rapidly in such a disk<sup>27</sup>, so that the dust opacity is reduced<sup>28</sup>. When the average size of the grains increases to  $\sim 10^{-2} \text{ cm}$ , the disk would be optically thin to stellar optical radiation as well as the infrared radiation emitted by the 10 K gas. However, the disk remains opaque to the ionizing photons. Thus the outward force on the ring will be dominated by the radiation pressure of the ionizing radiation, and is  $\sim 0.2 S_{47}$  times the gravitational attraction of the central star. Both the momentum flux and energy flux carried by the wind from a  $20 M_\odot$  star<sup>29</sup> are much less than those of the stellar radiation; therefore, the stellar wind cannot have a significant perturbation on the disk. (Note that the disk may encounter several passing field stars ( $v_* \approx 10 \text{ km s}^{-1}$ ) during its lifetime, but these brief encounters will not seriously disturb the disk dynamics.)

So far, we have seen that the protostellar disk model accounts naturally for the disk thickness and radius. But to be persuasive, it should also account for two other observed properties: (1) the enhanced nitrogen abundance, and (2), the  $10 \text{ km s}^{-1}$  radial expansion velocity.

The enhanced nitrogen abundance in the ring has a simple explanation: the inner boundary of the protostellar disk should be coated with a layer of gas expelled by the supernova progenitor. There is little doubt that the progenitor had a red giant stage, during which it lost nitrogen-enriched gas in a low-velocity wind, followed by a final blue giant stage lasting several thousand years. A fraction  $\sim z/R \approx 0.1$  of the total mass expelled by the red giant wind ( $\sim (1-10) M_\odot$ ) would be pasted on the inner rim of the disk, more than enough to account for the observed nitrogen enhancement. Thus, the inner boundary of the ring is actually mass expelled by the star. Note that the presence of the ring does not preclude the existence of an expanding spherical shell formed by the interaction of the red- and blue-giant winds of the progenitor, as in the original ejection hypothesis. Indeed, we think that the outer loops of the bipolar nebulosity seen around SN1987A<sup>5,6,30</sup> are parts of this shell illuminated by the X-ray flash of the supernova.

The low radial expansion velocity of the ring must be the result of a relatively recent impact of this shell with the inner rim of the disk. Suppose, for example, that the shell expands with constant velocity  $v_s \sim 50 \text{ km s}^{-1}$ . When it reaches the ring, it will 'paste' a mass  $\Delta M_s \approx \dot{M}_R (R/v_R) (z/R) \approx 10^{-2} M_\odot$  onto the ring, assuming that the red giant progenitor has wind with velocity  $v_R \approx 20 \text{ km s}^{-1}$  and mass loss rate  $\dot{M}_R \approx 10^{-5} M_\odot \text{ yr}^{-1}$ . The part of the shell that strikes the ring will transfer its momentum to the inner rim of the ring in an inelastic (radiative) collision, sufficient to accelerate the emitting  $\sim 0.04 M_\odot$  to  $\sim 10 \text{ km s}^{-1}$ . The impact must have been fairly recent—say,  $\lesssim 3,000 \text{ yr}$  before the supernova outburst; otherwise the shocked ring would have moved substantially. Indeed, the ragged appearance of the ring might be due in part to instabilities—'splashing' caused by the impact.

Is the ring around SN1987A the inner rim of a protostellar disk, or is it the waist of an hourglass-shaped bipolar nebula? In a few years, we should know the answer. The supernova envelope should strike the ring in about AD 1999  $\pm 3$  (refs 30–33). When it does, the shocked ring will suddenly become a very bright source of EUV and soft X-rays. This radiation will illuminate the (presently invisible) matter beyond the ring, causing it to fluoresce in narrow optical and ultraviolet emission lines. Then, with the Hubble Space Telescope, we shall see.  $\square$



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- Chevalier, R. A. *Nature* **332**, 514–516 (1988).
- Fransson, C. *et al. Astrophys. J.* **336**, 429–441 (1989).
- Wampler, E. J. & Richichi, A. *Astr. Astrophys.* **217**, 31–34 (1989).
- Lundqvist, P. & Fransson, C. *Astrophys. J.* **380**, 575–592 (1991).
- Crotts, A. P. S., Kunkel, W. E., & McCarthy, P. J. *Astrophys. J.* **347**, L61–L64 (1989).
- Wampler, E. J. *et al. Astrophys. J.* **382**, L13–L16 (1990).
- Jakobsen, P. *et al. Astrophys. J.* **369**, L63–L66 (1991).
- Panagia, N., Gilmozzi, R., Macchetto, F., Adorf, H.-M. & Kirshner, R. P. *Astrophys. J.* **380**, L23–L26 (1991).
- Crotts, A. P. S. & Heathcote, S. R. *Nature* **350**, 683–685 (1991).
- Luo, D. & McCray, R. *Astrophys. J.* **379**, 659–662 (1991).
- Wang, L. & Mazzali, P. A. *Nature* **355**, 58–61 (1992).
- Blondin, J. & Lundqvist, P. *Astrophys. J.* **405**, 337–352 (1993).
- Livio, M. & Soker, N. *Astrophys. J.* **329**, 764–779 (1988).
- Eriguchi, Y., Yamaoka, H., Nomoto, K. & Hashimoto, M. *Astrophys. J.* **392**, 243–248 (1992).
- Netzer, H. & Elitzur, M. *Astrophys. J.* **410**, 701–713 (1993).
- Podsiadlowski, Ph. *Nature* **350**, 654–655 (1991); *Publ. astr. Soc. Pacif.* **104**, 717–729 (1992).
- Meikle, W. P. S., Cumming, R. J., Spyromilio, J., Allen, D. A. & Mosbacher, B. in *SN 1987A and Other Supernovae* (eds Danziger, I. J. & Kjær, K.) 595–603 (European Southern Observatory, Garching, 1991).
- Kraft, R. P. in *Spectroscopic Astrophysics* (ed. Herbig, G. H.) 385–424 (Univ. California Press, Berkeley, 1970).
- Tereby, S., Shu, F. H. & Cassen, P. *Astrophys. J.* **286**, 529–551 (1984).
- Smith, B. A. & Terrell, R. J. *Science* **226**, 1421–1424 (1984).
- Goodman, A. A., Benson, P. J., Guller, G. A. & Myers, P. C. *Astrophys. J.* **406**, 528–551 (1993).
- Goldsmith, P. F. & Aquila, R. in *Protostars & Planets II* (eds Black, D. & Matthews, M. S.) 137–149 (Univ. Arizona Press, Tucson, 1985).
- Pringle, J. E. *Mon. Not. R. astr. Soc.* **238**, 37–41 (1988).
- Balick, B. *Astr. J.* **94**, 671–678 (1987).
- Ensmann, L. M. & Burrows, A. *Astrophys. J.* **393**, 742–755 (1992).
- Osterbrock, D. E. *Astrophysics of Gaseous Nebulae and Active Galactic Nuclei* (University Science Books, Mill Valley, California, 1989).
- Weidenschilling, S. J. & Cuzzi, J. N. in *Protostars and Planets III* (eds Levy, E. H. & Lunine, J. I.) 1031–1060, (Univ. Arizona Press, Tucson, 1993).
- Pollack, J. B., McKay, C. & Christofferson, B. *Icarus* **64**, 471–492 (1985).
- Chiosi, C. & Maeder, A. A. *Rev. Astr. Astrophys.* **24**, 329–375 (1986).
- Wang, L. & Wampler, E. J. *Astr. Astrophys.* **262**, L9–L12 (1992).
- Luo, D., McCray, R. & Slavin, J. *Astrophys. J.* **430**, 264–276 (1994).
- Suzuki, T., Shigeyama, T. & Nomoto, K. *Astr. Astrophys.* **275**, 883–894 (1993).
- Masai, K. & Nomoto, K. *Astrophys. J.* (in the press).

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## Blue-shifted oxygen lines and the clumpy ejecta of supernova 1993J

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MASSIVE stars that explode at the end of their lives (type II supernovae) have been generally thought to do so rather isotropically, but there have been very few near enough to our Galaxy to study in reasonable detail. Supernova 1993J, in the nearby galaxy M81, is the second-closest supernova since the invention of the telescope, which enables us to study its early evolution. Here we report the detection of blue-shifted oxygen lines only four months after the optical discovery of the supernova. Unlike the case of SN1987A (refs 1, 2), we suggest that this blue shift is not the result of dust formation, but probably arises because the photosphere of the supernova developed a complex, irregular structure near maximum light. The clumpy ejecta allows photons from oxygen on the near (blue-shifted) side of the photosphere, which would not be visible if the ejecta were smooth and isotropic, to escape and be detected, while the photons on the far (red-shifted) side of the supernova are still absorbed. This situation arises because of mixing of regions of different element groups<sup>3,4</sup>, and clearly shows the importance of instabilities during the supernova explosion.

The pre-maximum spectra of supernovae are modelled, at least to first order, by resonance scattering of continuum photons from a sharp photosphere<sup>5</sup>, where the early spectral features are characterized by the so called P-Cygni line profile, which has

a blue-shifted absorption trough and an emission peak at about zero velocity, and is produced by resonance scattering of continuum photons in an expanding atmosphere. Later, as a supernova reaches hydrogen recombination temperature, the photosphere remains at constant temperature and stays at roughly the same radius, therefore producing a plateau in its light curve. A dramatic change occurs when the photosphere retreats into the helium-rich layer and emission lines of heavy elements start to develop, and the supernova enters the photospheric-nebular transition phase.

The light curve of the supernova 1993J shows two maxima; the unusual initial peak and the absence of a clear plateau phase have led several groups to suggest that this supernova has a small mass of hydrogen in the atmosphere<sup>6,7</sup>, and that it is a type IIb supernova like SN1987K (refs 8, 9) which started as a type II supernova but eventually turned into a type Ib/c supernova. The early spectra show strong hydrogen Balmer lines, but optical and infrared helium lines are observed at about 23 days after shock break out<sup>10,11</sup>, consistent with the object being a type IIb supernova.

Figure 1 shows some spectra obtained with the Beijing Astronomical Observatory 2.16-m telescope. The presence of the forbidden oxygen lines, [O I] 6,300 Å doublet and [O I] 5,577 Å, is clear in the latest spectra of 8 August 1993. The emission feature in the vicinity of 5,500 Å is prominent in the spectra taken as early as when the supernova was in its second optical maximum. It was identified by Swartz *et al.*<sup>10</sup> as the [O I] 5,577 Å line in their 29 April 1993 spectra. This feature is not unique to SN1993J, however. Harkness *et al.*<sup>12</sup> find the [O I] 5,577 Å line present as early as 10 and 18 days past maximum in the spectra of type Ib supernova 1983N and 1984L, respectively. This identification was later disputed by Filippenko *et al.*<sup>13</sup>, who argue that the region is very complex and line identifications must be supported by detailed model calculations. Unambiguous [O I] emission began to form at about two months past maximum according to the latter authors<sup>13</sup>. We note that this feature reported here for SN1993J is remarkably similar to those in SN1983N and SN1984L, and may suggest further that SN1993J is similar to type Ib/c supernova at late times of evolution. However, as we have almost nightly observations of SN1993J, we want to point out here that this feature (which is clearly due to [O I] 5,577 Å in the 2 June 1993 spectra) can be traced back

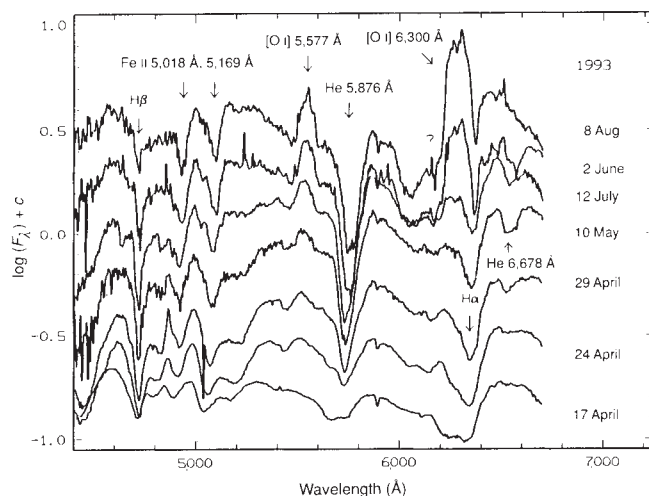
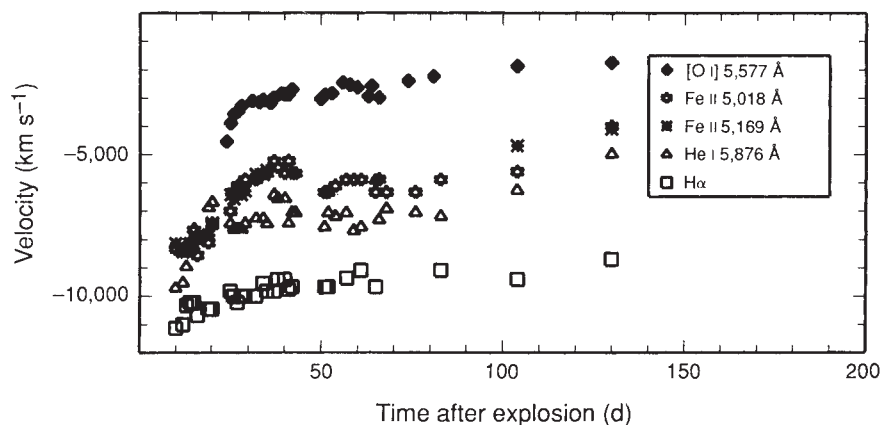


FIG. 1 The spectra of SN1993J. The fluxes  $F_\lambda$  are plotted on a logarithmic scale but the spectra are shifted arbitrarily for clarity. [O I] 5,577 Å and [O I] 6,300, 6,364 Å lines are present unambiguously in the spectra taken after 2 June 1993, but the features identified as these lines can be traced back smoothly to as early as 24 April 1993. The velocity of M81 is subtracted.

FIG. 2 The centroid velocity of the [O I] 5,577 Å line and the absorption minimum velocity for various P-Cygni line profiles, with the velocity to M81 subtracted. Note that the absorption minimum velocities are higher than, or close to, the expansion velocity of the oxygen shell. In the spherically symmetric model, this implies that most of the oxygen-rich material is inside an optically thick shell.



smoothly to as early as 24 April 1993 (Fig. 1), and that the presence of [O I] 5,577 Å line in the early spectra cannot be excluded.

The profile of the [O I] 6,300 Å line is corrupted by the absorption troughs of the H $\alpha$  P-Cygni profile, and we will thus base our discussions mainly on the [O I] 5,577 Å line. Figure 2 shows also the absorption trough velocities of some of the P-Cygni profiles and the peak velocities for the [O I] 5,577 Å line. It is surprising that the emission peaks of the [O I] lines are observed to be highly blue-shifted. Blue-shifted [O I] line profiles were also reported for SN1987A (ref. 1) showing up about 530 days after explosion, and was promptly taken as strong evidence for the onset of dust formation in the ejecta<sup>2</sup>. SN1993J is probably different from SN1987A. First, the observed blue shift is much larger than that in SN1987A, so that if this blue shift is due to dust formation, it would require an optical depth of  $\tau \gg 1.0$  to produce the almost completely blue-shifted line profiles. Such an optical depth would seriously influence the supernova light curve, and most of the supernova emission would appear in the infrared. This behaviour is, however, not observed. Second, the blue shift was observed at a much earlier date after explosion than in SN1987A. It is easy to verify that, in the oxygen-rich layer moving at typical velocities of 3,000 km s<sup>-1</sup>, the equilibrium temperature for dust particles is too high for them to condense or survive in the oxygen zone at the time of these observations.

Model supernova atmospheres demonstrate that the photospheric velocity can be measured directly from the absorption troughs of the weak Fe II 5,108 Å and 5,169 Å P-Cygni lines<sup>14,15</sup>. Hence, the velocities in Fig. 2 give quantitative estimates of the location of the photosphere at different dates. The ejecta is optically thick out to the photosphere, and emission lines develop only outside the photosphere. As the highest velocity observed for the oxygen line is <6,000 km s<sup>-1</sup>, and this velocity is lower than all the photospheric velocities measured from different P-Cygni line profiles, it follows that in the spherically symmetric model we get the seemingly paradoxical result that the [O I] lines formed inside the photosphere.

A simple explanation is that, during the transition from the photospheric phase to the nebular phase, the ejecta is partially optically thin. The nebular emissions from the approaching side could escape to the observer, while photons from the receding side are attenuated due to longer travelling distances. The overall line profile would then be blue-shifted, but the ejecta may still be spherically symmetric, as shown in the schematic plot in Fig. 3a. This is, however, an unlikely possibility. A continuum optical depth >1 is required to fit the observed line profiles, but this would contradict the assumption that the ejecta is getting optically thin. Also, the continuum optical opacity is roughly proportional to the electron density of the ejecta and therefore

is proportional to  $t^{-3}$ , where  $t$  is the time since explosion. A partially optically thin nebula will become optically thin in less than one dynamical timescale; by contrast the [O I] lines were observed to be blue-shifted from  $t \approx 40$  days to  $\approx 130$  days. It therefore seems that a partially optically thin nebula has difficulties in explaining the observations.

The above difficulties disappear if the ejecta is clumpy, as suggested by hydrodynamical calculations of supernova explosions<sup>3,4</sup>. The photosphere is unlikely to be spherically symmetric when it recedes to the helium shell, and is related to regions of higher ionization, such as those that are mixed with clumps of <sup>56</sup>Co, or are rich in light elements. Also, oxygen clumps can be driven up to the photosphere. Assuming that the velocity of the blue-shifted Fe I absorption feature indicates the location of the photosphere, the resulting picture is shown schematically in Fig. 3b. Whether or not there will be a sharply defined photosphere depends on whether the density profile close to the photosphere is steep. If the density gradient is large, there will be a well defined sharp photosphere, but it is likely to be irregularly structured. If the density profile is smooth, the nebula may be partially optically thin, and optically thick regions and optically thin clumps or filaments coexist in regions close to the photosphere. Several narrow components shown up in the spectra taken at different dates, and may suggest that the ejecta are indeed not uniform. Similar narrow components were also reported in SN1987A (ref. 16) and SN1985F (ref. 16), and were interpreted as arising from 'fingers' or clumps in the ejecta.

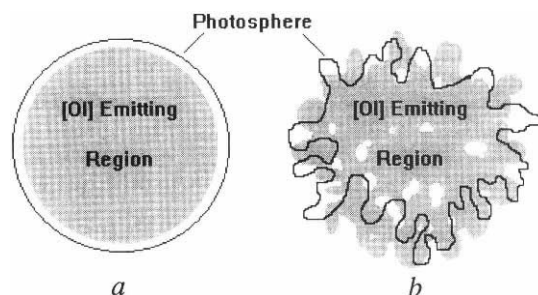


FIG. 3 Schematic plots of the supernova ejecta during the transition from photospheric phase to nebular phase. The shaded area shows the nebular line-emitting region, and the photosphere is shown as solid lines. *a*, The spherically symmetric case with a partially optically thin photosphere. *b*, Due to dynamical instability, the photosphere is irregularly structured, and [O I] emitting clumps can exist above the photosphere. Notice also that the boundary of the photosphere may not be sharply defined.



We expect the ejecta to become optically thin as time evolves, and eventually the blue shift of the [O I] lines will decrease. Figure 2 shows that the centre of the [O I] lines is indeed moving back to zero, but a significant blue shift is still apparent on 8 August 1993, implying that optically thick clumps still existed. The continuum opacity of the ejecta is  $6.65 \times 10^{-14} (n_e/10^{11}) \text{ cm}^{-1}$  for Thomson scattering and  $1.3 \times 10^{-14} (n_e/10^{11})^2 (\lambda/5,000)^2 (T/6,000)^{3/2} \text{ cm}^{-1}$  for free-free absorption, and both have optical depths of  $\sim 1$  for a dense clump of linear size  $> 2 \times 10^{13} \text{ cm}$  and electron density  $n_e \approx 10^{11} \text{ cm}^{-3}$ . Suppose the volume filling factor of the ejecta is  $f$ , with  $f=1$  being a uniform distribution. The density of each individual clump consequently increases by a factor of  $f$  of the average density  $\langle n_e \rangle$ . Assuming further that the size of each individual clump  $\sim \chi R$ , where  $R$  is the size of the photosphere and  $\chi$  is an arbitrary factor, we then find that the Thomson scattering optical depth of each individual clump  $\tau_c = \sigma_e \langle n_e \rangle \chi / f R$ . The average electron density  $\langle n_e \rangle$  is quite uncertain, but an order of magnitude estimate is given approximately by the requirement that on average the optical depth at the photosphere is  $\sim 1$  (or  $2/3$ ), that is,  $\sigma_e \langle n_e \rangle R \approx 1$ . The requirement that  $\tau_c > 1$  then yields  $\chi / f > 1$ . If, as suggested by hydrodynamical calculations<sup>3,4</sup>,  $\chi \approx 0.1$ , we then have  $f \leq 0.1$ . Note, however, that these relations are valid only in the photospheric-nebula-transition phase, which is indicated by the co-existence of P-Cygni profiles and nebular emission lines. We have also assumed that the density profile of the oxygen shell is not very steep, in keeping with the hydrodynamic calculations<sup>6</sup>.

Our model is testable by observations in the coming months. The blue-shifted [O I] lines will move back towards zero velocity as the photosphere retreats to the centre of the supernova envelope, while a definitive detection of dust formation will be sig-

nalled by a further blue shift of the [O I] lines. The peculiar behaviour of the [O I] lines in SN1993J is perhaps associated with the nature of its progenitor. Since this paper was submitted, we have learned (K. Nomoto, personal communication) that dynamical instability in the interface of helium and oxygen shell may have been very strong in the case of the explosion of the progenitor of SN1993J. The irregular nature of the ejecta may also provide some insights on the high continuum polarizations measured for SN1993J (ref. 18).  $\square$

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1. Danziger, I. J., Lucy, L. B., Bouchet, P. & Gouffes, C. in *Supernovae* (ed. Woosley, S. E.) 69–79 (Springer, New York, 1991).
2. Lucy, L. B., Danziger, I. J., Gouffes, C. & Bouchet, P. in *Structure and Dynamics of the Interstellar Medium* (eds Tenorio-Tagle, G., Moles, M. & Melnick, J.) 164–173 (Proc. IAU Colloq. 120, Lecture Notes in Physics, Springer, Berlin, 1989).
3. Arnett, W. D., Fryxell, B. & Müller, E. *Astrophys. J.* **341**, L63–L66 (1989).
4. Herant, M. & Benz, W. *Astrophys. J.* **387**, 294–308 (1992).
5. Branch, D., Doggett, J. B., Nomoto, K. & Thielemann, F.-K. *Astrophys. J.* **294**, 619–630 (1985).
6. Nomoto, K. et al. *Nature* **364**, 507–509 (1993).
7. Podsiadlowski, Ph., Hsu, J. J. L., Joss, P. C. & Ross, R. R. *Nature* **364**, 509–511 (1993).
8. Filippenko, A. V. *Astr. J.* **96**, 1941–1948 (1988).
9. Woosley, S. E. in *Supernovae* (ed. Woosley, S. E.) 202–212 (Springer, New York, 1991).
10. Swartz, D. A. et al. *Nature* **365**, 232–234 (1993).
11. Filippenko, A. V., Matheson, T., Ho, L. C. *Astrophys. J.* **415**, L103–L106 (1993).
12. Harkness, R. P. et al. *Astrophys. J.* **317**, 355–367 (1987).
13. Filippenko, A. V., Porter, A. C. & Sargent, W. W. *Astr. J.* **100**, 1575–1587 (1990).
14. Eastman, R. G. & Kirshner, R. P. *Astrophys. J.* **347**, 771–793 (1989).
15. Schmutz, W., Abbot, D. C., Russel, R. S., Hamann, W. R. & Wessolowski, U. *Astrophys. J.* **355**, 255–270 (1990).
16. Spyromilio, J., Stathakis, R. A. & Meurer, G. R. *Mon. Not. R. astr. Soc.* **263**, 530–534 (1993).
17. Filippenko, A. V. & Sargent, W. W. *Astrophys. J.* **345**, L43–L46 (1989).
18. Trammell, S. R., Hines, D. C. & Wheeler, J. C. *Astrophys. J.* **414**, L21–L24 (1993).

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## Synthesis and superconducting properties of the strontium copper oxy-fluoride

### $\text{Sr}_2\text{CuO}_2\text{F}_{2+\delta}$

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HIGH-PRESSURE synthesis has proved a useful technique for obtaining new, metastable copper oxide superconductors; for example, oxygen insertion into  $\text{Sr}_2\text{CuO}_3$  at 6 GPa (ref. 1) yields superconducting  $\text{Sr}_2\text{CuO}_{3.1}$ , with transition temperature  $T_c = 70 \text{ K}$ , in which the superconducting  $\text{CuO}_2$  layers are generated by pressure-induced oxygen migration from apical to equatorial sites. Although the simple structure and high transition temperatures make this family (general formula  $\text{Sr}_{n+1}\text{Cu}_n\text{O}_{2n+1+\delta}$ ) of interest, the stringent synthesis conditions limit its value for applications. Here we report that fluorine insertion into  $\text{Sr}_2\text{CuO}_3$  at ambient pressure causes related structural rearrangements to give superconducting  $\text{Sr}_2\text{CuO}_2\text{F}_{2+\delta}$  with a maximum  $T_c$  of 46 K. In this synthesis, the structural changes previously initiated by the thermodynamic effects of high pressure are induced chemically under ambient conditions. The result is a superconducting oxy-fluoride in which fluorine plays a dominant structural role, rather than merely being an electronic dopant as in  $\text{La}_2\text{CuO}_4\text{F}_x$  (ref. 2) and  $\text{Nd}_2\text{CuO}_{4-x}\text{F}_x$  (ref. 3).

High-pressure methods have been invaluable for the development of our understanding of copper oxide superconductors, as they have allowed the synthesis of metastable phases (such as

$\text{YBa}_2\text{Cu}_3\text{O}_8$  (ref. 4) and  $\text{YSr}_2\text{Cu}_3\text{O}_7$  (ref. 5)) which are difficult, or impossible, to obtain under ambient conditions. A recent observation<sup>1</sup> relates to the oxidation of  $\text{Sr}_2\text{CuO}_3$ , the structure of which can simply be derived from that of  $\text{La}_2\text{CuO}_4$  by removing chains of oxygen atoms from the  $\text{CuO}_2$  layers responsible for superconductivity in doped variants such as  $(\text{La}_{1-x}\text{Sr}_x)_2\text{CuO}_4$ , and  $\text{La}_2\text{CuO}_{4+x}$ . At 6 GPa and 800–900 °C,  $\text{Sr}_2\text{CuO}_3$  absorbs small amounts of oxygen and transforms structurally (by oxygen transfer from apical to equatorial positions around Cu) to a metastable superconducting phase  $\text{Sr}_2\text{CuO}_{3.1}$ ,  $T_c = 70 \text{ K}$  (ref. 1).  $\text{Sr}_3\text{Cu}_2\text{O}_{5+\delta}$ , the next member of this super-

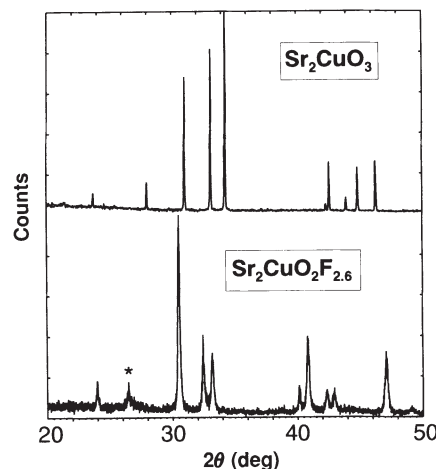


FIG. 1 Powder X-ray diffraction patterns ( $\text{Cu K}\alpha_1$ ) of  $\text{Sr}_2\text{CuO}_3$  before (top) and after (bottom) fluorination. The  $\text{SrF}_2$  impurity peak is marked\*.

TABLE 1 Refined structural data for  $\text{Sr}_2\text{CuO}_2\text{F}_{2.6}$ 

| Atom | Position | x          | y          | z          | B( $\text{\AA}^2$ ) | Cell occupancy |
|------|----------|------------|------------|------------|---------------------|----------------|
| Sr   | 8i       | 0          | 0          | 0.3680 (1) | 0.67 (2)            | 8              |
| Cu   | 4a       | 0          | 0          | 0          | 0.66 (3)            | 4              |
| O    | 8e       | 0.25       | 0.25       | 0          | 0.76 (3)            | 8              |
| F(1) | 32p      | 0.0358 (8) | 0.0483 (7) | 0.1802 (4) | 0.67 (7)            | 5.72 (5)       |
| F(2) | 32p      | 0.059 (2)  | 0.151 (2)  | 0.1802 (4) | 0.67 (7)            | 2.28 (5)       |
| F(3) | 32p      | 0.196 (2)  | 0.239 (2)  | 0.2212 (8) | 0.67 (7)            | 2.28 (5)       |

Unit cell dimensions;  $a = 5.394$  (1)  $\text{\AA}$ ,  $b = 5.513$  (1)  $\text{\AA}$ ,  $c = 13.468$  (3)  $\text{\AA}$ ; space group  $Fmmm$ ;  $R$  factors;  $R_{\text{wp}} = 3.7\%$ ,  $R_{\text{exp}} = 1.2\%$ ,  $R_1 = 7.2\%$ . Bond distances ( $\text{\AA}$ ): Cu–O, 1.928(1) ( $\times 4$ ); Cu–F(1), 2.45(1); Cu–O/F(2), 2.59(1); F(3)–F(1); 2.50(3), 2.65(3), 2.56(3); F(3)–F(2), 2.55(3).

conductor family, was reported to have  $T_c = 100$  K (ref. 1). Given that in both superconducting alloys and oxides the highest transition temperatures appear to occur in metastable phases<sup>6</sup>, the development of alternative chemical routes to simulate the thermodynamic effects of high pressures is of fundamental importance. Following earlier observations that oxygen can be inserted into the vacant sites in  $\text{Sr}_2\text{CuO}_3$  at low temperatures under moderate pressures<sup>7</sup>, we have examined the possibility of inserting elemental fluorine at relatively low temperatures and ambient pressure.

$\text{Sr}_2\text{CuO}_3$  (prepared from  $\text{SrCO}_3$  and  $\text{CuO}$ , using two heat treatments at  $950^\circ\text{C}$  for 16 h in air) was subjected to thermal treatments in a nickel furnace tube in a mixture of 10%  $\text{F}_2$ /90%  $\text{N}_2$  from which traces of HF had been removed by passing over NaF. Although moderate temperatures ( $400^\circ\text{C}$ ) were found to yield predominantly  $\text{SrF}_2$  owing to its high thermodynamic stability, at  $210^\circ\text{C}$  this reaction product was minimized (estimated at  $\sim 5$ –10% by X-ray diffraction). For treatments of 15 min at  $210^\circ\text{C}$ , a new orthorhombic ( $Fmmm$ ) phase was formed with unit-cell dimensions implying a close structural relationship to  $\text{La}_2\text{CuO}_4$ :  $a = 5.394$  (1),  $b = 5.513$  (1),  $c = 13.468$  (3)  $\text{\AA}$  (Fig. 1). Typically, a 200-mg sample of  $\text{Sr}_2\text{CuO}_3$  in a nickel boat was heated to  $210^\circ\text{C}$  in dry  $\text{N}_2$ , subjected to a

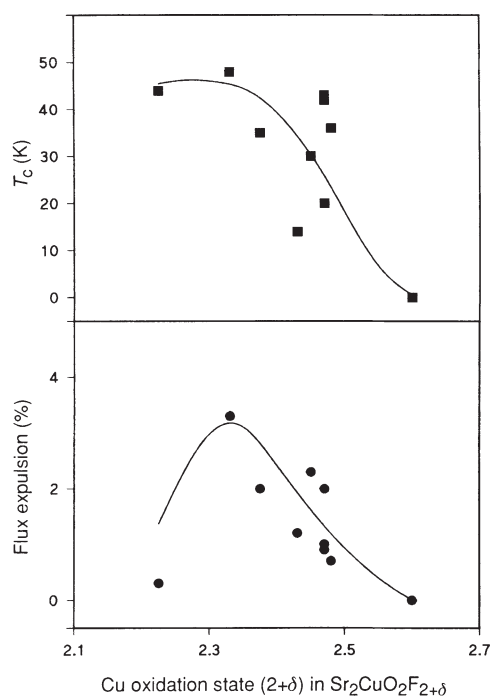


FIG. 2 Variation of  $T_c$  (top) and superconducting fraction (bottom) with copper oxidation state for  $\text{Sr}_2\text{CuO}_2\text{F}_{2+\delta}$ . The lines are a guide to the eye.

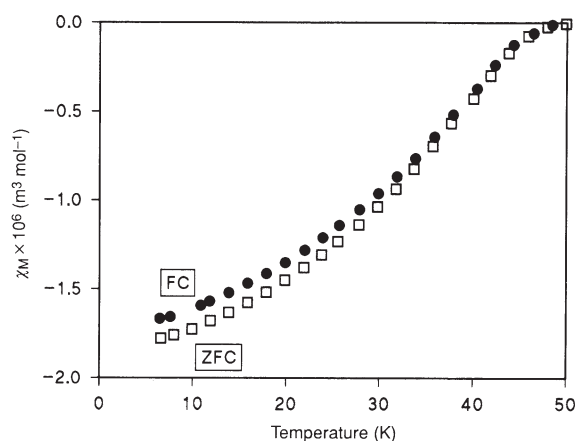


FIG. 3 Variation of molar susceptibility of  $\text{Sr}_2\text{CuO}_2\text{F}_{2.33}$  with temperature for both field-cooled (FC) and zero field-cooled (ZFC) experiments.

flowing  $\text{F}_2/\text{N}_2$  atmosphere for 15 min at this temperature, and allowed to cool in  $\text{N}_2$  or air after flushing all  $\text{F}_2$  from the system. The F content was estimated by precipitation titrations using thorium nitrate solution with methyl thymol blue indicator<sup>8</sup> and comparison with standard solutions containing similar cation concentrations. The mass loss during thermogravimetric reduction ( $\text{H}_2/\text{N}_2$  mixture at  $930^\circ\text{C}$  to form  $\text{SrF}_2$ ,  $\text{SrO}$ ,  $\text{Cu}$ ) followed by rapid analysis by X-ray diffraction of the  $\text{SrF}_2$ : $\text{SrO}$  ratio in the products (by comparison with standard mixtures) provided two additional independent estimates of the F:O ratio in the material. The Cu oxidation state was evaluated by iodometric titration. After small corrections for the  $\text{SrF}_2$  contamination (8 wt%), all results were consistent with the composition  $\text{Sr}_2\text{CuO}_2\text{F}_{2.6}$  (formal Cu oxidation state +2.6), which implies significant fluorine substitution for oxygen, in addition to fluorine insertion.

In view of the instability of  $\text{Sr}_2\text{CuO}_2\text{F}_{2.6}$  at sintering temperatures, resistance measurements were not attempted and its superconducting properties were examined magnetically with a d.c. SQUID magnetometer, using a field of  $10^{-3}$  T. Some samples were superconducting but with small ( $<1\%$ ) superconducting volume fractions; low-temperature reductions resulted in fluorine loss to give  $\text{Sr}_2\text{CuO}_2\text{F}_{2+\delta}$ , with no discernible structural change but improved superconducting properties. Samples were therefore annealed for up to 3 h in  $\text{N}_2$  (temperatures up to  $330^\circ\text{C}$ ,  $0.4 \leq \delta \leq 0.6$ ) or for 15 min in a 10%  $\text{H}_2/\text{N}_2$  mixture (temperatures up to  $220^\circ\text{C}$ ,  $0.2 \leq \delta \leq 0.4$ ). The superconducting properties ( $T_c$  and percentage flux expulsion) are shown in Fig. 2 in relation to the Cu oxidation state, and the susceptibility for the sample with  $T_c = 46$  K is shown in Fig. 3. The calculated superconducting volume fractions are small for all samples; this is attributed to particle size effects as it is well established that the magnetization is reduced for powders comprising crystallites with dimensions comparable to the London penetration depth<sup>9</sup>. This interpretation is supported by the broad X-ray diffraction peaks for  $\text{Sr}_2\text{CuO}_2\text{F}_{2.6}$  (Fig. 1), which indicate a mean crystallite size of only  $\sim 700$   $\text{\AA}$ , and the small divergence between field-cooled and zero field-cooled susceptibilities (Fig. 3). The data in Fig. 2 suggest that  $T_c$  and the superconducting volume fraction may show a broad maximum at a formal Cu oxidation state of +2.2 to +2.3, which is similar to other copper oxide superconductors. The wide range of oxidation states for which superconductivity is observed is thought to reflect composition variations, which are to be expected due to the low temperatures of the synthesis conditions.

Although neither X-ray nor neutron diffraction can differentiate O and F ions, bulk structural characteristics were investigated using powder neutron diffraction data collected on the



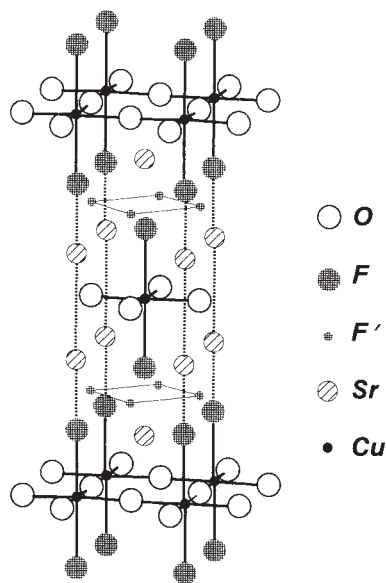


FIG. 4 Simplified structure of  $\text{Sr}_2\text{CuO}_2\text{F}_{2+\delta}$  showing idealized apical (F) and interstitial (F') sites.

POLARIS diffractometer in the ISIS facility, Rutherford Appleton Laboratory, to aid accurate location of the light atoms. The 4-g sample comprised the products from 15 consecutive fluorinations. Although structure refinement based on a simple  $\text{La}_2\text{CuO}_4$ -type model produced an unacceptably high and anisotropic temperature factor for the apical anions ( $B_{11} = 6 \text{ \AA}^2$ ,  $B_{22} = 23 \text{ \AA}^2$ ,  $B_{33} = 0 \text{ \AA}^2$ ) when considered as a single site, splitting this site into two positions was more satisfactory and resulted in sensible isotropic thermal parameters. This feature is similar to that observed in  $\text{La}_2\text{CuO}_{4+\delta}$  (refs 10, 11) and suggests the presence of interstitial F ions, consistent with the formula  $\text{Sr}_2\text{CuO}_2\text{F}_{2+\delta}$ . Inclusion of such ions near the ideal  $(\frac{1}{4}, \frac{1}{4}, \frac{1}{4})$  position produced a stable refinement. Refinements suggested that the interstitial site (F(3)), which would have four unacceptably short contacts ( $\sim 2.15 \text{ \AA}$ ) to neighbouring F ions in an undistorted structure, is displaced towards one of its neighbours which in turn shifts by  $\sim 0.6 \text{ \AA}$  to the F(2) site. Although the defect model is slightly different from those proposed for  $\text{La}_2\text{CuO}_{4+\delta}$  (refs 10, 11), one of which involves a very short O–O distance<sup>10</sup>, it is chemically plausible and avoids all close F–F contacts. However, the quality of the data and correlation effects did not allow accurate refinement of F(2) and F(3) site occupancies independently, and other defect models are possible. For the simple model proposed, the F(3) and F(2) occupancies are equal and were fixed accordingly in the final refinement, which suggested the composition  $\text{Sr}_2\text{CuO}_2\text{F}_{2.57(2)}$  (Table 1). Bond valence sum calculations<sup>12</sup> suggested a Cu oxidation state of +2.23 (using  $\text{Cu}^{2+}$  values for the parameter  $r_0$  as described in ref. 12), which is lower than the expected value of +2.6. Madelung energies were calculated<sup>13</sup> for different distributions of O and F atoms in the parent  $\text{Sr}_2\text{CuO}_2\text{F}_2$ , and indicated a substantial preference for apical F ( $11,072 \text{ kJ mol}^{-1}$ ) rather than equatorial F ( $10,138 \text{ kJ mol}^{-1}$ ) and provided strong support for the presence of only apical F. The structure of  $\text{Sr}_2\text{CuO}_2\text{F}_{2.6}$  is shown in simplified form in Fig. 4.

This study has revealed the new oxy-fluoride  $\text{Sr}_2\text{CuO}_2\text{F}_2$  and demonstrated its ability to support superconductivity. The evidence that F avoids the equatorial sites clearly demonstrates the potential role of oxy-fluorides for forming new high temperature copper oxide superconductors; superconductivity in  $\text{Sr}_2\text{CuO}_2\text{F}_2$  has been induced by incorporating interstitial F. The partial replacement of the apical  $\text{F}^-$  ions by  $\text{O}^{2-}$  could provide an alternative means of anionic chemical control of the electron carrier

density, in analogous fashion to Sr and Ba substitutions for La in  $\text{La}_2\text{CuO}_4$ . Of greater significance is the demonstration that the F site preference can promote interchange of O and F ions to create the structural features necessary to support superconductivity in phases which are otherwise unsuited, for example  $\text{Sr}_2\text{CuO}_3$ . This phenomenon, which has previously been observed only at very high pressures<sup>1</sup>, should result in the formation of other new superconducting phases by adopting similar synthetic strategies. □

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- Hiroi, Z., Takano, M., Asuma, M. & Takeda, Y. *Nature* **364**, 315–317 (1993).
- Tissue, B. M., Cirillo, K. M., Wright, J. C., Daeumling, M. & Larbalestier, D. C. *Solid St. Commun.* **65**, 51–54 (1988).
- James, A. C. W. P., Zahurak, S. M. & Murphy, D. W. *Nature* **338**, 240–241 (1989).
- Karpinski, J., Kaldis, E., Jilek, E., Rusiecki, S. & Bucher, B. *Nature* **336**, 660–662 (1988).
- Okai, B. *Jap. J. appl. Phys.* **29**, L2180–L2182 (1990).
- Sleight, A. W. *Chemtronics* **2**, 116–119 (1987).
- Lobo, R. C., Berry, F. J. & Greaves, C. J. *Solid St. Chem.* **88**, 513–519 (1990).
- Belcher, R. & Nutten, A. J. *Quantitative Inorganic Analysis* 3rd edn 281 (Butterworths, London, 1970).
- Campbell, A. M., Blunt, F. J., Johnson, J. D. & Freeman, P. A. *Cryogenics* **31**, 732–734 (1991).
- Challout, C. et al. *Physica C* **158**, 183–191 (1989).
- Radaelli, P. G. et al. *Phys. Rev. B* **48**, 499–510 (1993).
- Brown, I. D. & Altermatt, D. *Acta Crystallogr. B* **41**, 244–247 (1985).
- Weenk, J. W. & Harwig, H. A. *J. Phys. Chem. Solids* **38**, 1047–1054 (1977).

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## Charge-transfer states in dense hydrogen

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THE electronic properties of hydrogen, from the gas phase to the solid state, are fundamental to our understanding of the chemical bond<sup>1</sup>. The strong covalent bond of diatomic hydrogen persists in low-density condensed phases, where the molecules interact very weakly through van der Waals forces<sup>2</sup>. At very high densities, molecular bonding has long been predicted to give way to a monatomic and presumably metallic lattice<sup>3</sup>. At intermediate densities, intermolecular interactions are expected to increase; however, the relative strengths of the intermolecular and intramolecular interactions, and their effect on physical and chemical properties, have received comparatively little attention theoretically. Recent diamond-anvil-cell studies have revealed a range of unexpected phenomena in solid hydrogen at these densities<sup>4</sup>. Here we show that marked changes in the infrared and Raman spectra of the intramolecular stretching modes (vibrons)<sup>5</sup> with increasing pressure can be interpreted in a manner analogous to the behaviour of organic charge-transfer salts at ambient pressure, including those exhibiting pressure-induced neutral-to-ionic transitions<sup>6,7</sup>. Increased molecular overlap in dense hydrogen leads to symmetry breaking, which makes possible charge-transfer states between adjacent  $\text{H}_2$  molecules. The consequent changes in bond strength and in vibron frequencies are evident in the spectra. These findings present a new picture of dense hydrogen and highlight the advantages of a localized, 'chemical' description of the bonding.

The behaviour of hydrogen at megabar pressures ( $>100 \text{ GPa}$ ) is currently the subject of intense experimental and theoretical

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research<sup>4,8</sup>. Recent theoretical studies of H<sub>2</sub> at such pressures have focused on molecular orientation, band structure, or the lowest-energy crystal structure, using for example density functional approaches<sup>9–13</sup> and quantum simulations<sup>14</sup>. Vibrational spectra at high pressure indicate vibronic or electron-phonon coupling effects that have not been previously considered. The vibrational spectrum contains information about the higher energy excitonic transitions which are inaccessible by conventional high-pressure optical techniques<sup>15,16</sup>.

We focus on electronic information obtained from high-pressure measurements of the molecular vibron, which is derived from the stretching mode at 4,161 cm<sup>-1</sup> of the isolated molecule. The decrease in both the Raman and infrared vibron frequencies with increasing pressure above 30 GPa (ref. 17) and 140 GPa (ref. 18), respectively, suggest a gradual weakening of the bond (Fig. 1). The splitting  $\omega_R - \omega_{IR}$  provides an estimate of intermolecular interactions, which in turn allow us to rationalize the difference in pressure at which the frequency decrease begins for the Raman and infrared vibrons<sup>14,17</sup>. Discontinuous changes of the vibron frequencies in both the Raman<sup>19,20</sup> and infrared<sup>5</sup> spectra are important signatures of a major phase transition in the molecular solid at 150 GPa (phase III)<sup>4</sup>. Moreover, the infrared measurements demonstrate that there is a striking increase in vibron absorption by three orders of magnitude at the 150-GPa transition<sup>5</sup>. The abrupt increase in absorption tracks the pressure-temperature phase boundary of the transition, which is also characterized by changes in rotational (or librational) infrared bands<sup>4</sup>. The strong infrared absorption is remarkable because the vibrational excitation is Raman-active but infrared forbidden in the isolated molecule.

A convenient measure of the strength of absorption of electromagnetic radiation in materials is the classical oscillator strength  $f$ , which can be regarded as the effective number of electrons active in the transition<sup>21</sup>. Determination of  $f$  requires the evaluation of the integrated absorption, as shown in Fig. 2 for the principal infrared vibron  $Q_1(J)_{IR}$ . Low-pressure studies indicate that the low infrared intensity below 150 GPa is associated with weak intermolecular interactions which increase monotonically with density<sup>2,18,22</sup>. Infrared-active vibrations are possible in elemental solids of the proper symmetry<sup>23</sup>, but symmetry analyses

provide no information on the origin or strength of the absorption. The marked increase in  $f$  at 150 GPa is clearly evident in Fig. 2. Moreover, the result shows that  $f$  continues to increase with density well above the phase transition.

Charge-transfer (CT) processes in hydrogen have been suggested in some previous studies<sup>17,24,25</sup> but never examined in detail. Conventional CT compounds—ion-radical organic crystals—are now well understood<sup>26</sup>. Such compounds include planar  $\pi$ -electron donor ( $D$ ) and acceptor ( $A$ ) molecules which stack face-to-face, with sufficient overlap at  $\sim 3.3$  Å separation to have an intense CT excitation polarized along the stack<sup>27</sup>. The most extensively studied  $D$ s and  $A$ s have  $D_{2h}$  molecular symmetry, with infrared-forbidden  $a_g$  vibrations. Yet these totally symmetric modes often dominate<sup>28</sup> the infrared spectra in CT and ion-radical crystals and reflect a variety of packing motifs and oxidation states<sup>26</sup>. The  $a_g$  modes are coupled to charge-fluctuations along the stack arising from the Mulliken CT integral  $t$ , which is a measure of excited-state mixing and borrowing of intensity from the CT band<sup>26,27</sup>. We note that an inter-stack spacing of 3.3 Å is more than twice a carbon-carbon bond length; the nine-fold reduction of the H<sub>2</sub> molar volume around 150 GPa (ref. 29) leads to an intermolecular separation of  $\sim 1.9$  Å, again slightly more than twice the H<sub>2</sub> bond length of 0.74 Å. There is small but significant electron overlap in both cases, as we show below for hydrogen.

The principal ideas<sup>30</sup> in molecular crystals can be illustrated by dimers such as  $DA$ ,  $D^+D^+$ , or  $A^-A^-$ . Representative orientations of the corresponding H<sub>2</sub>H<sub>2</sub> dimer is shown in Fig. 3. The repulsive ground state potential for the dimers has been obtained from high-level *ab initio* calculations<sup>31</sup>. Another reason for starting with dimers is the lack of accurate structural and rotational information for hydrogen in the 150 GPa range. The molecular ground state  $|gg\rangle$  of non-overlapping dimers has precisely two electrons for each H<sub>2</sub>. As the molecules overlap, we consider CT states with excitation energy  $\hbar\omega_{CT}$

$$|CT_{\pm}\rangle = (|H_2^+H_2^-\rangle \pm |H_2^-H_2^+\rangle)/\sqrt{2} \quad (1)$$

The Mulliken CT integral  $t = \langle gg|H|CT_{\pm}\rangle$  mixes the two even states according to the coefficient  $c \approx t/\hbar\omega_{CT}$  and generates a

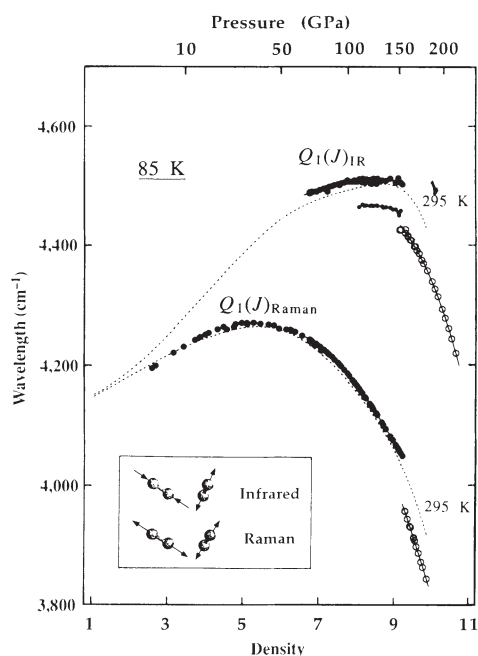


FIG. 1 Density dependence of the frequencies of the infrared and Raman vibrons of solid hydrogen ( $Q_1(J)_{IR}$  and  $Q_1(J)_{Raman}$ , respectively). Here  $Q_1$  refers to a  $\Delta v=1$ ,  $\Delta J=0$  transition, where  $v$  and  $J$  are vibrational and rotational quantum numbers; this terminology is strictly valid only at lower densities where the molecules exist in free rotational states<sup>4</sup>. Raman scattering excites the even (symmetric) combination of the stretching modes, whereas the odd (out-of-phase) combination is excited in infrared absorption, as shown in the inset for two molecules in arbitrary orientation (for example, the unit cell of the rotationally disordered hexagonal close packed (h.c.p.) structure). The densities were calculated from the pressure using the X-ray equation of state<sup>29</sup>;  $\rho_0 = 0.0435$  mol cm<sup>-3</sup>, the density of the solid at zero pressure and low temperature ( $T \rightarrow 0$  K). The small filled circles near 4,465 cm<sup>-1</sup> correspond to the frequency of the weak infrared vibron that appears at 110 GPa and 85 K (phase II; refs 4, 5). The larger filled circles correspond to the vibrons in the lower-pressure phases (I and II); the empty circles are the vibron frequencies in the high-pressure phase (III). The dashed line shows the vibron shifts at 295 K (phase I). Phases II and III have lower symmetry than h.c.p. (as indicated for example, by the two infrared vibrons above 110 GPa), although the continuity of phonon modes from low pressure and across the transitions suggests the crystal structures with respect to the molecular centres are related to h.c.p.<sup>18,38</sup>. Before the 150-GPa transition, the molecules adopt either librational states (consistent with partial orientational order) of free rotational states, both of which are consistent with low-frequency measurements<sup>5,18,38</sup>. Combination bands involving excitation of the vibron and rotational or librational states change at the 150-GPa transition (that is, on entering phase III)<sup>4</sup>. Phases I, II and III meet at a triple point near 130 K (ref. 4).



dipole-allowed transition to  $|CT_{-}\rangle$  (where  $H$  is the full hamiltonian of the system)<sup>26,30</sup>. Closed-shell  $D$  and  $A$  molecules lead to ion-radical dimers  $D^{+}D^{+}$  or  $A^{-}A^{-}$  in which the highest occupied molecular orbital (HOMO) or lowest unoccupied molecular orbital (LUMO) are singly occupied, whereas CT dimers admix  $DA$  and  $D^{+}A^{-}$ . An  $H_2H_2$  dimer involves closed-shell species for which CT is not normally significant. Charge fluctuations given by equation (1) are symmetric, and each  $H_2$  acts as both donor (by way of the HOMO) and acceptor (by way of the LUMO). The antibonding LUMO is odd under inversion, and this has important implications for the magnitude of  $t$ .

We consider next the implication of an admixture  $c$  of  $|CT_{+}\rangle$  in the  $|gg\rangle$  ground state. The CT transition moment for displacing a charge by  $r \approx 2 \text{ \AA}$ , the approximate intermolecular distance at 150 GPa, is  $c_{\text{CT}}$ , where  $c$  is the mixing coefficient defined above and  $e$  the electron charge. The resulting oscillator strength of the putative CT excitation is  $f_{\text{CT}} = \hbar \omega_{\text{CT}} c^2 e^2 r^2 = 2c^2$ , assuming  $\omega_{\text{CT}}$  is near the effective optical gap (oscillator frequency  $\omega_1 \approx \omega_{\text{CT}} \approx 4 \text{ eV}$ ) at 150 GPa inferred from dielectric measurements<sup>15,32</sup>. The vibron data can be used to provide a direct estimate of  $f_{\text{CT}}$ . Because the  $H_2^+$  bond is weaker and  $H_2^-$  is unbound<sup>33</sup>, the bond becomes weaker and the symmetric stretching mode frequency  $\omega$  shifts to a lower value  $\omega'$  above the phase transition. The dimensionless electron-phonon coupling constant<sup>26,34</sup> is  $\lambda = (\omega^2 - \omega'^2)/\omega^2$ , which reduces to  $2\delta\omega/\omega$  for small shifts. We have  $\lambda \approx 0.04$  for a  $100 \text{ cm}^{-1}$  Raman shift at the transition; typical values for  $\lambda$  in open-shell molecular and polymeric systems are 0.1–0.3. Dynamic coupling<sup>30</sup> of the dimer's odd linear combination of stretches and  $|CT_{-}\rangle$  leads to a similar red shift of the infrared vibron. Because  $\lambda$  couples the infrared and CT transitions, the infrared shift and intensity

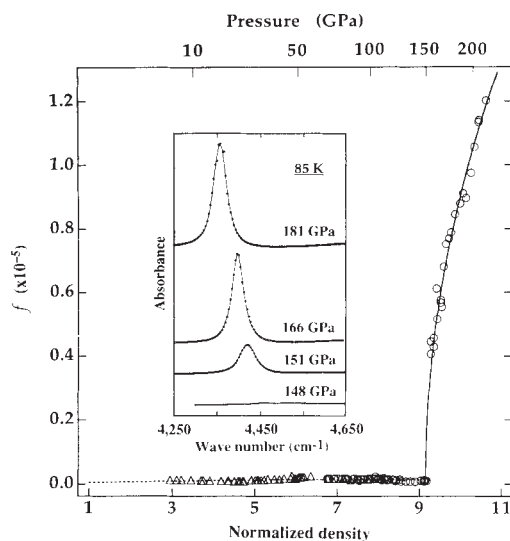


FIG. 2 The density dependence of the oscillator strength  $f$  for the infrared vibron in solid hydrogen. (Density normalized as in Fig. 1.) The oscillator strength<sup>21</sup> is defined as  $f = 4.315 \times 10^{-9} \int \epsilon(\nu) d\nu$  where  $\epsilon(\nu)$  is the molar extinction coefficient (in  $\text{cm}^{-1} \text{ mol}^{-1} \text{ l}$ ) given by  $\epsilon(\nu) = \log(I_0/I)/(cl)$ , where  $c$  is the concentration in  $\text{mol l}^{-1}$ ,  $l$  is the sample thickness (2  $\mu\text{m}$ ), and  $I$  and  $I_0$  are the intensities of the transmitted and incident light, respectively. The circles show the results of the analysis of data measured at 85 K, and the solid line is a quadratic fit to the points above  $P_{\text{tr}}$ , the pressure of the phase transition (150 GPa). The triangles are room-temperature data measured below the transition (refs 22, 24); the dashed line is a fit to all of the lower pressure measurements. The inset shows the actual change in absorption of the infrared vibron from 148 to 181 GPa at 85 K. The lines are Voigt profile fits to the peaks used to calculate integrated absorption, which is given by the area under the curves.

borrowed from the CT transition are related. Their oscillator strengths are<sup>30,35</sup>

$$f_{\text{CT}} = f_{\text{IR}} \lambda^{-1} (\omega_{\text{CT}}/\omega_1 R)^2 = f_{\text{IR}} (\omega/2\delta\omega) [1 + (2\delta\omega/\Gamma)^2] \quad (2)$$

where  $\Gamma$  is the full width at half maximum of the infrared absorption, and the lowest-order contributions in  $\delta\omega/\Gamma$  are kept. At the onset of the 150-GPa transition,  $\Gamma \approx 25 \text{ cm}^{-1}$ , which gives a  $f_{\text{CT}}/f_{\text{IR}}$  ratio of  $1.3 \times 10^3$ . An  $f_{\text{IR}}$  of  $10^{-5}$  (Fig. 2) leads to  $f_{\text{CT}} \approx 0.013$  (a relatively intense absorption<sup>21</sup>). Moreover,  $\omega_{\text{CT}}$  closely tracks  $\omega_1$  with increasing density  $\rho$ :  $d\omega_{\text{CT}}/d(\rho/\rho_0) \approx -1.3 \text{ eV}$  and  $d\omega_1/d(\rho/\rho_0) = -1.2 (\pm 2) \text{ eV}$  (refs 15,32). The close correspondence between  $\omega_{\text{CT}}$  and  $\omega_1$  suggests that the electronic transition that largely controls the dispersion of the refractive index in the high-pressure phase<sup>15</sup> is a CT-type excitation.

Our use of vibrational data at the 150-GPa transition is just the reverse of standard treatments of CT compounds, where  $\omega_{\text{CT}}$  and  $f_{\text{CT}}$  are measured directly and related to electron-phonon coupling constants for many  $a_g$  modes in the material<sup>26</sup>. The simplicity of  $H_2$ , with a single totally symmetric ( $a_g$ ) vibration, makes it possible to infer a CT excitation  $\omega_{\text{CT}}$  from the vibrational spectrum. This approach is particularly important for diamond-cell studies because electronic transitions above  $\sim 3 \text{ eV}$  are inaccessible by conventional spectroscopic techniques at these pressures due to absorption by the anvils<sup>15,16</sup>. Further details and extended calculations, including application to  $H_2$  lattices and separation of  $\delta\omega = \omega - \omega'$  into ground and excited-state contributions, will be published elsewhere<sup>35</sup>.

The hypothesis that  $|CT_{+}\rangle$  becomes admixed at  $P_{\text{tr}} = 150 \text{ GPa}$  rationalizes the equal  $\Delta\omega_{\text{IR}} \approx \Delta\omega_{\text{R}}$  and enhanced infrared intensity. But why should  $t$  be negligible below  $P_{\text{tr}}$ ? The Mulliken CT integral  $t = \langle gg|H|CT_{+}\rangle$  reduces<sup>26</sup> to a Coulomb exchange integral  $\langle \text{HOMO}|\text{H}|\text{LUMO} \rangle$  between the two molecules, and this one-electron integral is extremely sensitive to the relative orientations of the molecules (Fig. 3). Because the LUMO is odd under inversion,  $t$  vanishes rigorously for the X or P geometries and is maximized for the L geometry. Moreover, the odd  $H_2^+$  electronic function requires an odd rotational molecular

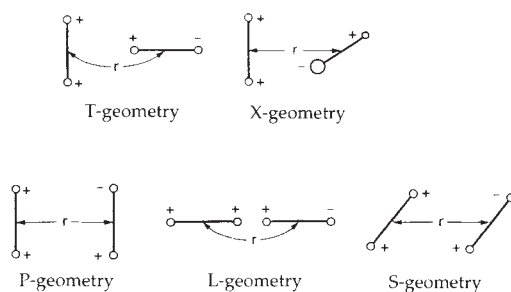


FIG. 3 Representative configurations of  $H_2H_2$  dimers for the present CT calculations. The ++ indicates the phase of the highest occupied molecular orbital (HOMO), which serves as the electron donor and has even symmetry; the +- denotes the phase of the lowest occupied molecular orbital (LUMO), the acceptor orbital which has odd symmetry. Only one of several possible  $H_2^+H_2^-$  charge fluctuations for a given dimer configuration is shown. The CT states in equation (1) hold for P (parallel), S (slanted) and L (linear) dimers, which have an inversion centre at the midpoint  $r/2$  between molecules. The CT integral  $t = \langle \text{HOMO}|\text{H}|\text{LUMO} \rangle$  vanishes by symmetry for X (pseudo-tetrahedron) and P, is finite for S, and is maximized for L, which, however, has the strongest ground-state repulsions<sup>31</sup>. For S and L, the two molecules of the dimer have equal donor and acceptor properties; that is, electronic fluctuations are symmetric. In the T configuration, on the other hand, the molecules indicated by ++ and +- must be the donor and acceptor, respectively, and CT in the other direction vanishes. When there is no inversion symmetry, the coefficients of the two terms in equation (1) differ.

wavefunction to satisfy the Pauli principle<sup>2</sup>. Intermolecular forces between nonoverlapping molecules mix in even rotational functions (even quantum number  $J$ )<sup>3,6</sup>, while the charge fluctuations described above mix odd  $J$ s. This leads to the possibility that the onset of CT is associated with the mixing of even and odd  $J$ s in the system at 150 GPa. Such a symmetry breaking could be associated with either an orientational ordering transition or a change in ordering (a reorientational transition<sup>11</sup>), both of which are consistent with changes in infrared vibron combination bands at the transition<sup>4</sup>.

Of the possible ordered configurations for the high-pressure phase, structures with approximately T and P neighbours are predicted by density-functional calculations<sup>9–13</sup>. In structures derived from hexagonal close-packed,  $H_2$  tilts of  $\sim 60^\circ$  relative to the  $c$  axis are calculated for alternate  $ab$  planes<sup>11,12</sup>. Although the L configuration has the strongest ground-state repulsions<sup>31</sup>, its stabilization by local CT effects may give physical insight into the origin of the chain structures noted in recent large-scale quantum simulations of the dense molecular solid<sup>14,37</sup>. In contrast to fluctuations involving  $H_2^+H_2^-$  molecular ions described here, it has been suggested<sup>21</sup> that  $H^+$  and  $H^-$  ions are formed at 150 GPa; however, such disproportionation is expected to give rise to much larger changes in vibrational and optical properties than are observed at this pressure<sup>5,15,16,19,20,38</sup>. A significant feature of our approach is that its predictions are amenable to direct experimental tests. For example, it may be possible to access the electronic (excitonic) states predicted from the CT model using newly developed high-pressure multiphoton spectroscopic techniques<sup>39</sup>. The large effect of molecular orientation on CT, like that predicted previously for metallization<sup>9–11</sup>, point to a critical coupling of orientational, structural and electronic properties in the system at  $\sim 150$  GPa.  $\square$

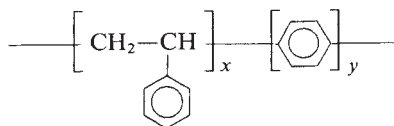
## Self-organized honeycomb morphology of star-polymer polystyrene films

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AN important challenge in the preparation of porous polymer membranes for technological applications is to control both the size distribution and the relative positions of the pores. We have found a way to generate polymer films with an essentially monodisperse pore size, in which the pores are organized spontaneously into periodic hexagonal arrays. The films, which are 10–30  $\mu\text{m}$  thick, are produced by evaporating solutions of star-shaped polystyrene or polystyrene–polyparaphenylene block copolymers in carbon disulphide under a flow of moist gas. Empty spherical cells, about 0.2–10  $\mu\text{m}$  in diameter, appear spontaneously in a hexagonal array, and the cells are open at the film surface. The use of star polymers, or of polymeric micelles, seems to be essential for obtaining this morphology. These membranes might find application in controlled release of drugs or other bioactive species, or as materials with useful optical properties, moulds or scaffolding for forming ordered microstructures, and model substrates for surface science.

We observed the formation of this regular morphology (Fig. 1) when preparing polystyrene–polyparaphenylene (PS–PPP) block copolymer films for radiation scattering experiments. The copolymers have the following formula:



They are aggregated both in solution and in the solid state into quasi-spherical micelles with an insoluble PPP core surrounded by a PS shell<sup>5</sup> (Fig. 2). The size of these cores measured by neutron scattering is in the range 3–15 nm. It was observed that some films of these copolymers, prepared by evaporating carbon disulphide ( $\text{CS}_2$ ) solutions, present a quite unusual aspect and morphology. They produce bright interference colours, and spot diffraction patterns, characteristic of an hexagonal packing, were seen from laser-light scattering. Microscopic studies revealed a hexagonal array of empty cells (Fig. 1). On the assumption that the shape of the aggregates plays a role in this process we prepared films from star-shaped polystyrene macromolecules<sup>6</sup> (Fig. 2). The same morphology was observed (Fig. 1d). On the other hand, linear polystyrenes never lead to regular morphologies.

In practice the  $\text{CS}_2$  solutions ( $2\text{--}100\text{ g l}^{-1}$ ) are spread and dried on a flat surface in a flow of moist air or moist inert gas. No special morphology was observed in either dry or methanol-saturated conditions. Even in humid conditions, a minimum evaporation rate is required to get the honeycomb morphology. This quick evaporation induces cooling of the solution layer. Several organic solvents were tried but only solutions in carbon disulphide led to the ordered microstructure.

A typical optical micrograph of a PS–PPP film prepared from a  $10\text{ g l}^{-1}$  solution, is presented in Fig. 1a. A regular hexagonal arrangement of holes is visible. Electron scanning micrographs (Fig. 1b, c) confirm this observation and show that holes have a regular size. Cross-sectioning the film in Fig. 1a showed that the whole film is made of layers of empty spheres. With an adhesive tape it is easy to remove the superficial skin and to see

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- Heitler, H. & London, F. Z. *Phys.* **44**, 455–472 (1927).
- van Kranendonk, J. *Solid Hydrogen* (Plenum, New York, 1983).
- Wigner, E. & Huntington, H. B. J. *chem. Phys.* **3**, 764–770 (1935).
- Mao, H. K. & Hemley, R. J. *Rev. mod. Phys.* **66**, 671–692 (1994).
- Hanfland, M., Hemley, R. J. & Mao, H. K. *Phys. Rev. Lett.* **70**, 3760–3763 (1993).
- Takaoka, Y. et al. *Phys. Rev.* **B36**, 3884–3887 (1987).
- Hanfland, M., Brillante, A., Girlando, A. & Syassen, K. *Phys. Rev.* **B38**, 1456–1461 (1988).
- Ascroft, N. W. in *Frontiers of High-Pressure Research* (eds Hochheimer, H. D. & Etters, R. D.) 115–129 (Plenum, New York, 1991).
- Garcia, A., Barbee, T. W., Cohen, M. L. & Silvera, I. F. *Europhys. Lett.* **13**, 355–360 (1990).
- Chacham, H. & Louie, S. G. *Phys. Rev. Lett.* **66**, 64–67 (1991).
- Kaxiras, E., Broughton, J. & Hemley, R. J. *Phys. Rev. Lett.* **67**, 1138–1141 (1991).
- Nagara, H. & Nakamura, T. *Phys. Rev. Lett.* **68**, 2468–2471 (1992).
- Surh, M. P., Barbee, T. W. & Mailhot, C. *Phys. Rev. Lett.* **70**, 4090–4093 (1993).
- Hohl, D., Notoiu, V., Ceperley, D. M. & Martin, R. M. *Phys. Rev. Lett.* **71**, 541–544 (1993).
- Hemley, R. J., Hanfland, M. & Mao, H. K. *Nature* **350**, 488–491 (1991).
- Eggert, J. H. et al. *Phys. Rev. Lett.* **66**, 193–196 (1991).
- Sharma, S. K., Mao, H. K. & Bell, P. M. *Phys. Rev. Lett.* **44**, 886–888 (1980).
- Hanfland, M., Hemley, R. J., Mao, H. K. & Williams, G. P. *Phys. Rev. Lett.* **69**, 1129–1132 (1992).
- Hemley, R. J. & Mao, H. K. *Phys. Rev. Lett.* **61**, 857–860 (1988).
- Lorenzana, H. E., Silvera, I. F. & Goettel, K. A. *Phys. Rev. Lett.* **63**, 2080–2083 (1989).
- Kauzmann, W. *Quantum Chemistry* 568–583 (Academic, New York, 1957).
- Mao, H. K., Xu, J. & Bell, P. M. in *High Pressure in Science and Technology* (eds Homan, C., MacCrone, R. K. & Whalley, E.) 327–331 (North-Holland, New York, 1984).
- Zallen, R. *Phys. Rev.* **173**, 824–832 (1968).
- Loubeyre, P., LeToullec, R. & Pinceaux, J. P. *Phys. Rev.* **B45**, 12844–12853 (1992).
- Baranowski, B. *Polish. J. Chem.* **66**, 1637–1640 (1992).
- Bozio, R. & Pecile, C. in *Spectroscopy of Advanced Materials* (eds Clark, R. J. H. & Hester, R. E.) 1–86 (Wiley, New York, 1991).
- Soos, Z. G. & Klein, D. J. in *Molecular Association* (ed Foster, R.) 1–109 (Academic, London, 1975).
- Rice, M. J. *Solid St. Commun.* **31**, 93–98 (1979).
- Hemley, R. J. et al. *Phys. Rev.* **B42**, 6458–6470 (1990).
- Painelli, A. & Girlando, A. J. *chem. Phys.* **84**, 5655–5671 (1986).
- Ree, F. H. & Bender, C. F. J. *chem. Phys.* **71**, 5362–5375 (1979).
- Hemley, R. J. et al. in *Strongly Coupled Plasma Physics* (eds Van Horn, H. M. & Ichimaru, S.) 3–10 (Univ. Rochester Press, Rochester, New York, 1993).
- Amaya-Tapia, A., Cisneros, C. & Russek, A. *Phys. Rev.* **A34**, 2591–2598 (1986).
- Mele, E. J. in *Handbook of Conducting Polymers* (ed. Skotheim, T.) 795 (Dekker, New York, 1986).
- Soos, Z. G., Eggert, J. H., Hemley, R. J., Hanfland, M. & Mao, H. K. J. *chem. Phys.* (submitted).
- Janssen, W. B. L. M. & van der Avoird, A. *Phys. Rev.* **B42**, 838–848 (1990).
- Maddox, J. *Nature* **364**, 483 (1993).
- Hemley, R. J., Mao, H. K. & Shu, J. F. *Phys. Rev. Lett.* **65**, 2670–2673 (1990).
- Lipp, M. J. & Daniels, W. B. *Phys. Rev. Lett.* **67**, 2810–2813 (1991).

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the internal honeycomb structure (Fig. 1*d*). The thickness of the walls of the spheres is 0.1–0.2  $\mu\text{m}$ . Depending on experimental conditions (solution thickness and concentration) one or several layers are obtained. An example of a monolayer is shown in Fig. 3. These morphologies were observed also by atomic force microscopy (Fig. 4).

The length of PS sequences in PS-PPP copolymers, or the length of PS arms in star-like PS, influences the morphology: regular morphologies were observed for relative molecular masses ( $M_r$ ) between 1,500 and 50,000 (1.5K and 50K). The size of the spheres increases with  $M_r$ . For higher values, the regularity disappears gradually. The number of arms of star-shaped PS plays also a role, in that the regularity increases with the number of arms. Further attempts to obtain the same morphology with other homopolymers and copolymers without polystyrene sequences were unsuccessful.

These data suggest that three conditions are required to produce this unusual honeycomb morphology: use of homopolymers or block copolymers star-shaped PS, use of  $\text{CS}_2$  as solvent, and condensation at the solution surface under moist conditions.

The mechanism of formation of this new regular morphology involves a combination of complicated thermodynamics and transport phenomena. Any explanation must remain somewhat speculative at this stage, but some features may be related to

the classical 'phase inversion' process (commonly used in the production of microporous polymeric membranes<sup>7</sup>) as follows. (1) The special thermodynamic behaviour of PS macromolecules in  $\text{CS}_2$  solution. A gelation process takes place when the polymer concentration is high enough<sup>8</sup>. The gelation point depends on the temperature and it was recently reported that the gel point temperature of star-shaped PS is high compared to the values for linear polymers<sup>9</sup>. (2) The process by which the polymer solution comes into contact with a non-solvent fluid. Water is deposited at the solution surface by condensation and is able to start a phase inversion through, presumably, a germination and growth process. The mechanism of formation of the morphology can then be described as follows: rapid evaporation increases the superficial concentration and induces a cooling of the solution surface, producing condensation of water. These three factors provoke a gelation process and a phase separation, with the formation of a polymer film at the solution surface (possibly around water droplets). Convection currents seen in the solution at the beginning of the evaporation process may be due to local variations of the superficial tension (that is, Marangoni convection). These currents may favour a regular stacking of the spheres that result from the demixing process. With the return to room temperature, the liquid pressure would create holes in the superficial spheres allowing the evaporation of sol-

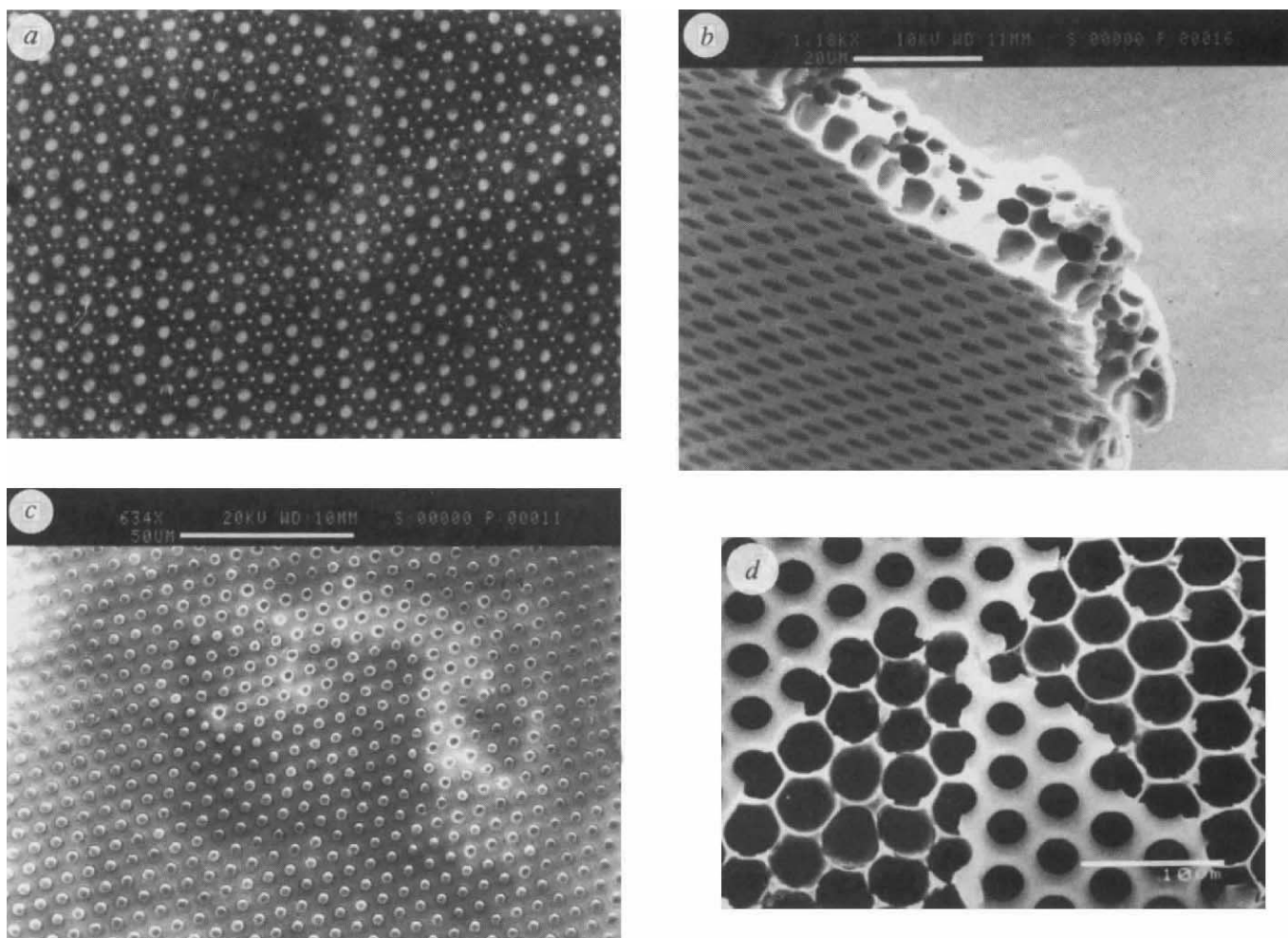


FIG. 1 *a*, Optical micrograph of the polystyrene-polyparaphenylene (relative molecular mass 30K–7K) block copolymer showing the regular arrangement of holes. Magnification,  $\times 272$ . *b*, *c*, Scanning electron micrographs showing the honeycomb morphology of the same film as shown in *a*. *b*, Sideways view; *c*, plan view. *d*, Scanning electron micro-

graph of a star polystyrene film showing the honeycomb morphology. (This polymer has seven branches, each having a relative molecular mass of 10K.) Part of the superficial skin has been removed to show the internal morphology. Scale bars: *b*, 20  $\mu\text{m}$ ; *c*, 50  $\mu\text{m}$ ; *d*, 10  $\mu\text{m}$ .

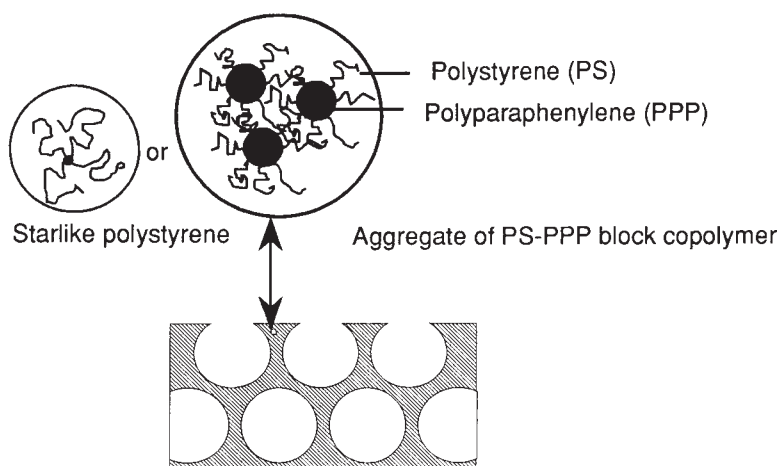


FIG. 2 Schematic cross-section of a regular microporous film prepared by evaporation of carbon disulphide solutions of homopolymer or block copolymer star-shaped polystyrene.

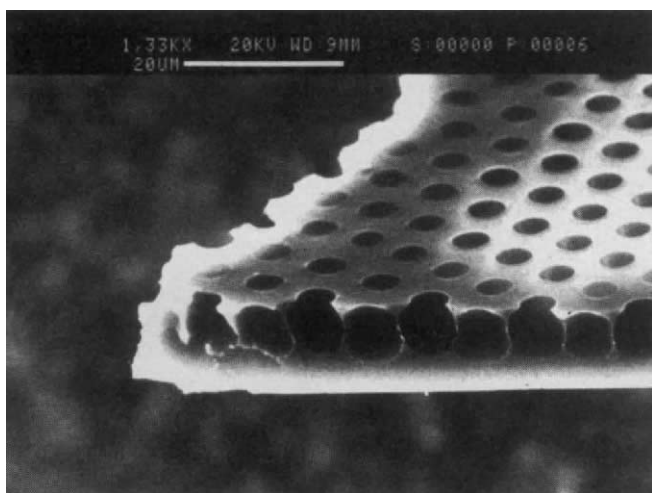


FIG. 3 Scanning electron micrograph showing a monolayer of pores in a PS-PPP copolymer film. Scale bar, 20  $\mu\text{m}$ .

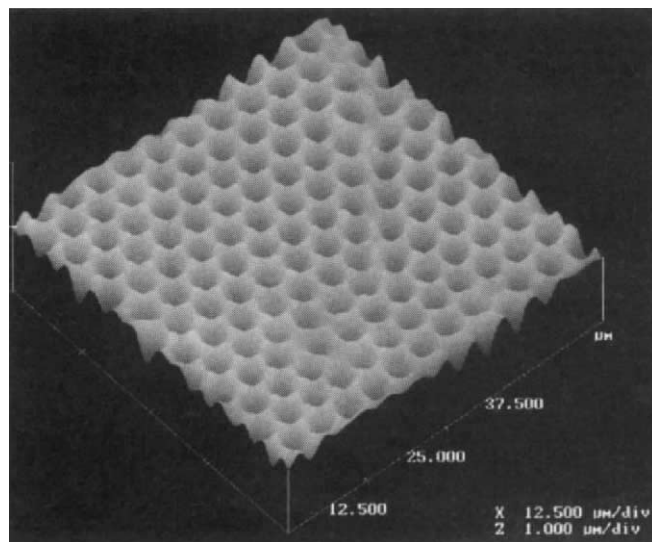


FIG. 4 Atomic force micrograph of the honeycomb morphology of a PS-PPP copolymer film. (Note that the z axis is compressed 12.5  $\times$  compared to the x and y axes: units are  $\mu\text{m}$ .)

vent and water. Within the framework of this model, star-shaped polymers play a major role, firstly in decreasing the solution viscosity, and secondly in favouring the gelation and phase separation. These effects are controlled by the number and the length of arms of the macromolecules.

It is quite remarkable that the polymer architecture is able to induce a complex regular morphology at a scale two or three orders greater than the molecule scale. Another notable aspect of this process is the formation of 'macro-crystals' of spherical pores with classical grain boundaries and point dislocations. (Fig. 4). The regularity of pore sizes, of the thickness of their walls and their optimized stacking are expected to improve considerably the mechanical properties and the efficiency of membranes made from this material: such improvements could be applied to give better control of the release of drugs or other bioactive species. The crystalline organization of the pores, with typical sizes close to the wavelengths of light, could be useful in optical applications. The organized open spheres at the film's surface could be used as micro-moulds for a controlled disper-

sion of materials or as scaffolding to induce other ordered morphologies. More generally these films could be used as models for surface science studies, such as wetting, adhesion and transport properties. It is expected that suitable experimental conditions could be found to obtain similar morphologies with other polymers. □

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1. Zhong, X. F. & François, B. *Synth. Metals* **29**, 35–40 (1989).
2. Zhong, X. F. & François, B. *Makromolek. Chem.* **192**, 2277–2291 (1991).
3. François, B. & Zhong, X. F. *Synth. Metals* **41–43**, 955–958 (1991).
4. Widawski, G., Rawiso, M. & François, B. *J. Chim. Phys.* **89**, 1331–1336 (1992).
5. Widawski, G. thesis, Univ. Strasbourg (1993).
6. Worsfold, D. J., Zilliox, J. G. & Rempp, P. *Can. J. Chem.* **47**, 3379–3385 (1969).
7. Kesting, R. E. in *Synthetic Polymer Membranes* Ch. 7 (Wiley, New York, 1985).
8. Gan, Y. S., Sarazin, D., Guenet, J. M. & François, J. *Polymer* **29**, 898–903 (1988).
9. Leshaini, N., Boudenne, N., Zilliox, J. G. & Sarazin, D. *Macromolecules* (in the press).

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# Convection in multicomponent silicate melts driven by coupled diffusion

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THE simplest convecting systems are driven by buoyancy forces arising from the density variations produced by the diffusion of a single quantity, usually heat. In such systems, the necessary condition for the onset of convection is that density decreases downwards. But the convection process can be very different if there are two or more diffusing quantities that affect the density<sup>1–5</sup>, a situation often encountered in geological settings. For example, multicomponent convection plays an important role in the convective evolution and differentiation of terrestrial magmas<sup>6–8</sup>. A typical terrestrial silicate magma is made up of a number of diffusing components, which not only have different diffusion rates but may also exhibit diffusive coupling—that is, the diffusive flux of one component can be strongly dependent on the spatial gradients of other components<sup>9–12</sup>. We have investigated convection in a geologically relevant fluid—a multicomponent silicate melt in the system CaO–Al<sub>2</sub>O<sub>3</sub>–SiO<sub>2</sub>—and we find that diffusive coupling plays a key role in promoting convection. Perhaps the most striking feature of these experiments is that convection can occur regardless of whether the density decreases or increases with depth.

Multicomponent convection due to diffusive coupling (represented below by off-diagonal terms in the diffusion matrix) is demonstrated using experiments in the system CaO–Al<sub>2</sub>O<sub>3</sub>–SiO<sub>2</sub> at 1,500 °C and 10 kbar. The experimental design we used for both interdiffusion and convection experiments is shown in Fig. 1. In those experiments in which we juxtaposed melts with compositions such that no convection occurs, we can use the interdiffusion profiles to determine the elements of the diffusion matrix,  $D_{ij}$ . These relate the mass fluxes  $J_i$  to concentration gradients  $\nabla C_j$  according to the general relation<sup>13</sup>

$$J_i = -\rho_0 \sum_{j=1}^{n-1} D_{ij} \nabla C_j \quad (1)$$

or specifically for our system,

$$J_{\text{CaO}} = -\rho_0 (D_{\text{CaO,CaO}} \nabla C_{\text{CaO}} + D_{\text{CaO,SiO}_2} \nabla C_{\text{SiO}_2}) \quad (2)$$

$$J_{\text{SiO}_2} = -\rho_0 (D_{\text{SiO}_2,\text{CaO}} \nabla C_{\text{CaO}} + D_{\text{SiO}_2,\text{SiO}_2} \nabla C_{\text{SiO}_2}) \quad (3)$$

where  $n$  in equation (1) is the number of components and  $\rho_0$  is the mean density. These equations are for the  $(n-1)$  independent fluxes with Al<sub>2</sub>O<sub>3</sub> considered a dependent component as it can be calculated given the concentrations of CaO and SiO<sub>2</sub>. Table 1 gives the values of the elements of the diffusion matrix derived from fitting interdiffusion profiles that were run in three different directions in composition space. The off-diagonal terms are not negligible, and we show below that the large negative off-diagonal term coupling silica flux to gradients in CaO plays an important role in explaining the existence and style of multicomponent convection in some of our experiments.

Our main interest is in situations that are convectively unstable. In the first experiment, we place a more dense melt above a less dense melt. This is a straightforward situation in which we expect simple overturning. This is exactly what we observe as shown in Fig. 2a. The concentration maps clearly show that the more dense melt is sinking along the left side of the section while the lighter melt is rising along the right. Notice that Fig. 2a also shows compositional variations in silica, despite its being initially uniform. This is due to the non-negligible off-

TABLE 1 Compositions and properties of molten CaO–Al<sub>2</sub>O<sub>3</sub>–SiO<sub>2</sub>

| Sample | CaO (wt%) | Al <sub>2</sub> O <sub>3</sub> (wt%) | SiO <sub>2</sub> (wt%) | Density (g cm <sup>-3</sup> ) |
|--------|-----------|--------------------------------------|------------------------|-------------------------------|
| CT-1   | 29.95     | 9.86                                 | 60.18                  | 2.653                         |
| CT-2   | 25.15     | 19.97                                | 54.87                  | 2.660                         |
| CT-3   | 20.18     | 15.35                                | 64.67                  | 2.600                         |
| CT-4   | 25.04     | 9.93                                 | 65.03                  | 2.614                         |
| CT-5   | 30.46     | 15.13                                | 54.41                  | 2.681                         |
| CT-6   | 20.01     | 19.85                                | 60.14                  | 2.618                         |

| Diffusion matrix | $D_{\text{CaO,CaO}}$ ( $\times 10^{-7}$ cm <sup>2</sup> s <sup>-1</sup> ) | $D_{\text{CaO,SiO}_2}$ ( $\times 10^{-7}$ cm <sup>2</sup> s <sup>-1</sup> ) | $D_{\text{SiO}_2,\text{CaO}}$ ( $\times 10^{-7}$ cm <sup>2</sup> s <sup>-1</sup> ) | $D_{\text{SiO}_2,\text{SiO}_2}$ ( $\times 10^{-7}$ cm <sup>2</sup> s <sup>-1</sup> ) |
|------------------|---------------------------------------------------------------------------|-----------------------------------------------------------------------------|------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|
|                  | 4.66                                                                      | 0.44                                                                        | -2.72                                                                              | 1.00                                                                                 |

The melt was at 1,500 °C and 10 kbar. The elements of the diffusion matrix were obtained from simultaneously fitting interdiffusion data between CT-1 and CT-6 (0.25 h duration), CT-2 and CT-4 (2 h), and CT-3 and CT-5 (2 h).

diagonal terms in the diffusion matrix (compare Table 1 and equation (3)).

Figure 2b is a concentration map for an experiment in which the same compositions used in Fig. 2a were reversed, so that the less dense melt was initially above the more dense melt. One might imagine this to be a stable situation, yet we again find that convection takes place. But now the structure of the flow is in the form of a 'finger' sinking along the axis of the cylindrical container. The negative buoyancy of the 'finger' is due mainly to depletion in silica caused by  $D_{\text{SiO}_2,\text{CaO}} < 0$  (Table 1). The flux of silica due to the off-diagonal term in the diffusion matrix is seen more clearly in Fig. 4, which shows profiles of CaO (Fig. 4a), Al<sub>2</sub>O<sub>3</sub> (Fig. 4b), and SiO<sub>2</sub> (Fig. 4c) at three different times, for experiments using the same arrangement of glasses as shown in Fig. 2b. At 0.25 hours convection has not yet significantly distorted the diffusive behaviour of the system, and we see that the interdiffusion of CaO and Al<sub>2</sub>O<sub>3</sub> has produced variations in the silica profile that can be interpreted in terms of a flux of silica driven by CaO gradients that is opposite to the direction

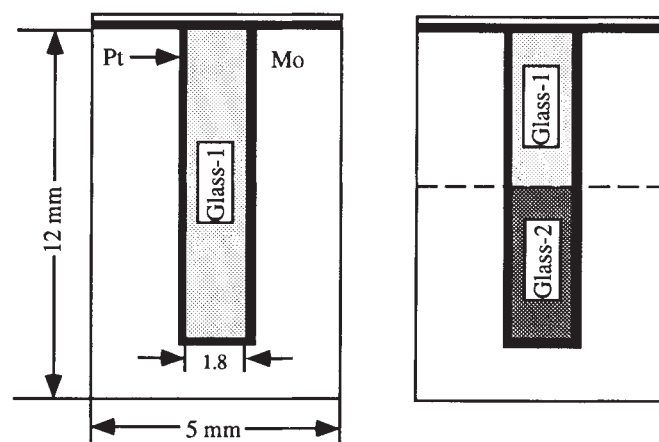


FIG. 1 Experimental design. Uniform starting materials (glasses 1 and 2, which are melts at run conditions) were first synthesized in a piston cylinder apparatus at 1,500 °C and 10 kbar using platinum-lined molybdenum capsules (left panel). Diffusion or convection couples were then formed by cutting the original capsules in half and then joining halves containing different glasses. The newly joined couple was then again placed in the piston cylinder apparatus at 1,500 °C and 10 kbar for the duration of the experiment (right panel). After quenching, the charge was sectioned longitudinally, polished and mounted for electron microprobe analysis. Previous experiments with similar capsules<sup>18</sup> show that the vertical temperature differences are small across the capsule ( $\leq 5$ –10 °C), and negligible in terms of their effect on the convection discussed in this Letter.

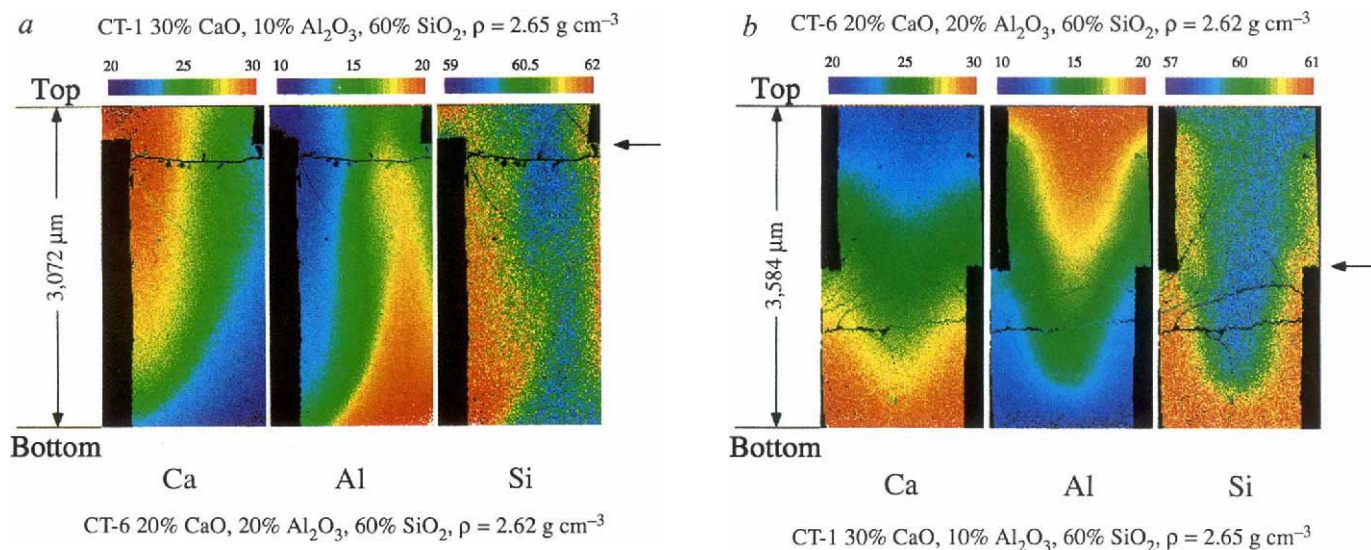


FIG. 2 False-colour X-ray intensity maps of the elements Ca, Al, and Si showing convection in the samples (Fig. 1). The ranges of the compositional variations (wt%) for each element are represented by the horizontal scale bars on top of the X-ray image. The black strips on the sides of each image are the container walls. Thin irregular dark lines are dilation cracks produced when the sample was quenched at the end of the run. The arrows on the right-hand side of the Si panel mark the location of the original interface. The step along the container wall is due to a small misalignment when joining capsules together. *a*, X-ray maps of the lower portion of the experimental charge, showing convective overturning in a 2-h run where a denser melt (CT-1,  $\rho = 2.65 \text{ g cm}^{-3}$ ) was placed above a less dense melt (CT-6,  $\rho = 2.62 \text{ g cm}^{-3}$ ). *b*, X-ray maps of the central portion of the charge showing convective finger instability in a 2-h run where the less dense melt (CT-6) was placed above the denser melt (CT-1). The X-ray intensities were collected using a Cameca SX-50 electron microprobe with an accelerating voltage of 15 kV, beam current of 100 nA and beam diameter of 5  $\mu\text{m}$ .

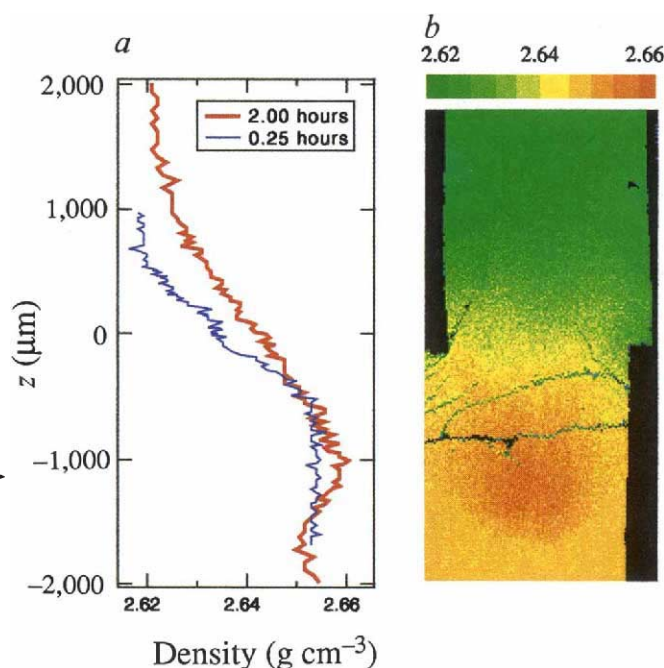


FIG. 3 *a*, Vertical density profiles (along the cylindrical axis  $z$ ) calculated using partial molar volumes from ref. 17 and the 0.25 and 2.0-h concentration data from Fig. 4. *b*, False-colour two-dimensional density map constructed from the concentration maps shown in Fig. 2*b*. The red portion of the density map shows the negative buoyancy associated with the downgoing finger seen in terms of compositions in Fig. 2*b*. The ranges of density variations ( $\text{g cm}^{-3}$ ) are shown by the horizontal scale bar.

of the flux of CaO itself. This is due to  $D_{\text{SiO}_2, \text{CaO}} < 0$ . The concentration profiles at 2.0 hours have become significantly distorted by convection, and thus can no longer be interpreted in terms of one-dimensional diffusion. Figure 3 shows a profile (Fig. 3*a*) and a two-dimensional map of density (Fig. 3*b*) constructed from the composition data shown in Figs 2*b* and 4. The density field at 2.0 hours clearly shows that the sinking finger is associated with a region of negative buoyancy due to depletion in  $\text{SiO}_2$ , and therefore that what we are seeing in the experiment is indeed a case of convection destabilized by an off-diagonal term in the diffusion matrix of the silicate melt.

A formalism already exists for predicting convective instability in multicomponent systems with non-zero off-diagonal terms in the diffusion matrix<sup>14, 15</sup>, and we have shown<sup>16</sup> that our experiments are perfectly consistent with such a stability analysis once

the cylindrical geometry of our experiments is taken into account. For present purposes we prefer to give a more phenomenological explanation of the cause of the fingering instability leading to the convection seen in Fig. 2*b*. The density–composition relation for the melts we used in this study can be expanded in a linear form using the partial molar volume data given in ref. 17.

$$\rho = 2.639(1 + 0.179(C_{\text{CaO}} - 0.25) - 0.209(C_{\text{SiO}_2} - 0.15)) \quad (4)$$

where the density is in  $\text{g cm}^{-3}$  and concentration is in weight fraction. Figures 3 and 4 show that by 0.25 hours, the flux of  $\text{SiO}_2$  drive by the evolving CaO gradient has produced a region with very little vertical density gradient at the level of the initial interface. Any local perturbation that now pushes the original interface downwards will push low-CaO melt into a region richer



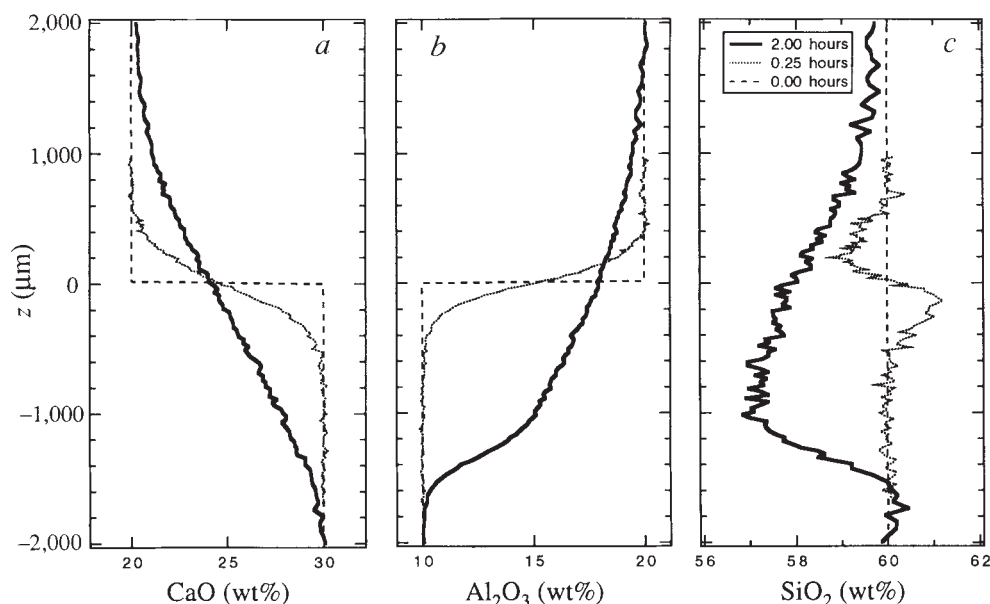


FIG. 4 a–c, Normalized concentration variations (in CaO,  $\text{Al}_2\text{O}_3$  and  $\text{SiO}_2$ , respectively) along the cylindrical axis ( $z$ ) in two runs of different duration. The initial concentration distributions are shown as dashed lines. The thin dotted lines are profiles along the axis of a 0.25-h run, whereas the thick lines are the profiles along the axis of the run shown in Fig. 2b. The starting compositions in the two runs differed slightly (by 0.1–1.0 wt%) and thus were rescaled to a common initial change in composition.

in CaO; this will have the effect of setting up horizontal gradients that will drive a flux of CaO into the perturbed region. Because  $D_{\text{SiO}_2, \text{CaO}} < 0$ , there will be an associated flux of  $\text{SiO}_2$  out of the perturbed region with the effect of increasing the density of the perturbed region, decreasing that of the surroundings, and thus producing buoyancy forces that lead to further sinking. The same line of argument leads one to conclude that an upward perturbation of the interface of the melt couple shown in Fig. 2b would also grow, and indeed we have run experiments showing such an upward-moving 'silica finger'.

Given the potential complexity of convection in multicomponent magmatic systems, the subject is still in an exploratory stage: what is needed is a better understanding of the causes and consequences of the various instabilities that can arise in such systems. The implication of the experiments presented here is that in order to fully understand convection in laboratory or natural magmatic systems, one needs to take into account not only that different density-affecting components of the melt can diffuse at different rates but also that these components are very likely to be coupled by off-diagonal terms in the diffusivity matrix. □

## Earliest known tetrapod braincase and the evolution of the stapes and fenestra ovalis

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*ACANTHOSTEGA gunnari*, from the Upper Devonian (Famennian) of East Greenland, is the most primitive known tetrapod, and retains many fish-like characters<sup>1,4</sup>. I report here the discovery of further well preserved specimens that show the earliest known tetrapod braincase, and shed light on the history of the tetrapod ear region. The fenestra ovalis is shown to be derived directly from the vestibular fontanelle<sup>5,6</sup>, a hole in the sidewall of the braincase of fishes seen in their embryology and in primitive fossil fish adults. The hole is not a uniquely tetrapod character<sup>7,8</sup>. A specialized auditory fenestra ovalis may have evolved more than once among tetrapods. As in other tetrapods, the stapedial footplate of *Acanthostega* fitted into the fenestra ovalis, but instead of being free to vibrate as part of an ear, was firmly held there, forming a major component of the braincase wall. It was the only component linking the otic capsule to the palate. Though the stapes may have carried muscles operating a spiracular valve, the new material suggests that it was not a mobile component of the skull as previously suggested<sup>4</sup>. The stapes, spatially replacing parts of the fish braincase including the process carrying facets for the hyomandibular articulation<sup>9</sup>, has a footplate which incorporates both heads of the sarcopterygian hyomandibula.

The neurocranium is represented in several specimens of *Acanthostega*, the most complete being that of University Museum of Zoology, Cambridge, specimen UMZC T1300a. This somewhat dorsoventrally compressed braincase lacks only the anterior parts of the sphenethmoid (Fig. 1). It is preserved with the exoccipitals and basioccipital in position, visible in three dimensions except where they are obscured by the impacted atlas intercentrum. Good information also comes from an almost uncrushed specimen with the epipterygoids and pterygoids in position, and associated with both stapes (Geological Museum, Copenhagen, specimen MGUH f.n. 1300a). It lacks parts of the opisthotics,

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1. Stommel, H., Arons, A. B. & Blanchard, D. *Deep-Sea Res.* **3**, 152–153 (1956).
2. Turner, J. S. A. *Rev. Fluid Mech.* **6**, 37–56 (1974).
3. Huppert, H. E. & Turner, J. S. J. *Fluid Mech.* **106**, 299–329 (1981).
4. Turner, J. S. A. *Rev. Fluid Mech.* **17**, 11–44 (1985).
5. Schmitt, R. W. A. *Rev. Fluid Mech.* **26**, 255–285 (1994).
6. Sparks, R. S. J., Huppert, H. E. & Turner, J. S. *Phil. Trans. R. Soc. A* **310**, 511–534 (1984).
7. Huppert, H. E. & Sparks, R. S. J. A. *Rev. Earth planet. Sci.* **12**, 11–37 (1984).
8. McBirney, A. R. *Igneous Petrology* (Jones & Bartlett, London, 1993).
9. Hofmann, A. W. in *Physics of Magmatic Processes* (ed. Hargraves, R. B.) 315–417 (Princeton Univ. Press, 1980).
10. Sugawara, H., Nagata, K. & Goto, K. S. *Metall. Trans.* **8B**, 605–612 (1977).
11. Kubicki, J. D., Muncill, G. E., & Lasaga, A. C. *Geochim. cosmochim. Acta* **54**, 2709–2715 (1990).
12. Kress, V. C. & Ghiorso, M. S. *Geochim. cosmochim. Acta* **57**, 4453–4466 (1993).
13. de Groot, S. R. & Mazur, P. *Non-Equilibrium Thermodynamics* (Dover, New York, 1962).
14. Baines, P. G. & Gill, A. E. J. *Fluid Mech.* **37**, 298–306 (1969).
15. McDougall, T. J. J. *Fluid Mech.* **126**, 379–97 (1983).
16. Liang, Y. Paper presented at AGU Chapman Conf. on Double-Diffusive Convection 3–6 November 1993, Scottsdale, Arizona, USA.
17. Lang, R. A. & Carmichael, I. S. E. *Geochim. cosmochim. Acta* **51**, 2931–2946 (1987).
18. Ayers, J. C., Brennan, J. B., Watson, E. B., Wark, D. A. & Minarik, W. G. *Am. Miner.* **77**, 1080–1086 (1992).

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and the basioccipital has in places become uncoupled from the basisphenoid. Together with partial information from other specimens, a confident restoration can be made (Fig. 2a, b).

A key feature revealed by the *Acanthostega* braincase is the position of the suture linking the basioccipital (parachordal) with the basisphenoid (trabecular) regions of the neurocranium. In all other tetrapods except *Ichthyostega*<sup>5</sup> this region is covered by the backward growth of the parasphenoid. In *Acanthostega*, the parasphenoid is very limited in extent, confined to a narrow groove between the basipterygoid processes, allowing the suture to be traced dorsally until it reaches the otic capsule. It defines the posterodorsal extent of the basisphenoid, revealing that neither the basisphenoid nor the parasphenoid contributed to the margin of the 'fenestra ovalis'. Incorporation of the basiparasphenoid into the margin has been assumed to distinguish the tetrapod fenestra ovalis from the fish vestibular fontanelle, though what constitutes a tetrapod fenestra ovalis, independent of the stapes that it houses, has not been formally defined. Bordered anteriorly only by the otic capsule and basioccipital, the fenestra ovalis of *Acanthostega* has the same relationships to the braincase as both the fish vestibular fontanelle and the embryonic basicapsular fenestra<sup>10</sup>. What was previously only a minority view<sup>5,6</sup> is corroborated by this material. A true fenestra ovalis, with evidence of association with hearing, for example the separate existence of a fenestra rotunda, or a position entirely confined within the otic capsule in the adult, probably arose with the differentiation of high-frequency hearing mechanisms several times in different lineages<sup>11,12</sup>.

Because there was no otic process of the epipterygoid contacting the otic capsule, the only contacts between braincase and palate were through the stapes and at the basal articulation. The latter contact exhibits a synovial joint surface with a bifaceted basipterygoid process. Found also in loxomatids<sup>13</sup>, colosteids<sup>14</sup>, embolomeres<sup>15</sup> and early temnospondyls<sup>16</sup>, this

appears to be a basal tetrapod character derived within the group and thus a synapomorphy, but characteristic only of primitive forms. It cannot be used to define a subgroup of tetrapods<sup>17</sup>.

The reconstructed braincase bears closest resemblance to that of early ray-finned fish such as *Moythomasia*<sup>6</sup>, *Mimia*<sup>6</sup> and *Pteroniscus*<sup>18</sup> or the acanthodian *Acanthodes*<sup>19</sup>, rather than to that of the osteolepiform *Eusthenopteron*<sup>5</sup> (Fig. 2). It would be illuminating to compare the *Acanthostega* braincase with that, currently undescribed, of *Panderichthys*, a lobe-finned fish with no intracranial joint<sup>20</sup>, more closely related to tetrapods than is *Eusthenopteron*<sup>20</sup>, or with that of a juvenile osteolepiform. Early actinopterygians and acanthodians arguably approach the primitive gnathostome condition, with tetrapods appearing to be plesiomorphic or at least paedomorphic in braincase construction. If correct, my interpretation suggests that a plesiomorphic or juvenile character has been exploited by tetrapods for use in a specialized structure.

Another unexpected feature of the *Acanthostega* braincase is the relatively large size of the vestibular fontanelle, and of the stapedia footplate, which fills it almost completely. Most specimens of *Acanthostega* braincases retain associated stapes, suggesting they were firmly incorporated into the braincase wall. Although the dorsal margin of the fontanelle formed by the otic capsule is smooth and rounded and somewhat thickened, the portions formed by the occipital component remain incompletely ossified. The dorsal margin is here taken to indicate the rim of an open (not cartilage-filled) vestibular fontanelle, in which the stapedia footplate was positioned. The footplate contributed such a large proportion of the sidewall of the braincase (Fig. 2a) that it may be considered a component of the braincase, rather than simply an element which fitted into a hole there.

The stapedia footplate is formed from two sub-circular facets set at a slight angle to one another, in anterior view separ-

FIG. 1 *Acanthostega gunnari*, braincase. a, b, Interpretive drawings of ventral and right lateral views of University Museum of Zoology, Cambridge, specimen UMZC T1300a; inset, medial view of stapes showing footplate, from Geological Museum, Copenhagen, specimen MGUH field number 1300a. c, d, Photographs of UMZC T1300a, same views as a and b. Ventral surface of skull table, light mechanical stipple; vestibular fontanelle, heavy mechanical stipple; broken bone, hatched. Scale bar, 10 mm. Abbreviations for this figure and for Fig. 2: atlas ic, atlas inter-centrum; bocc, basioccipital or equivalent area; bptpr, basipterygoid process or equivalent; bsphen, basisphenoid or equivalent area; exocc, exoccipital; hyom facet, hyomandibular facet; jug canal, jugular canal; lat ot fiss, lateral otic fissure; occ arch, occipital arch; orb art, foramen for orbital artery; ot cap, otic capsule; psphen, parasphenoid; sphet, sphenethmoid; stap for, stapedia foramen; v cran fiss, ventral cranial fissure; v cran sut, ventral cranial suture; v font, vestibular fontanelle.

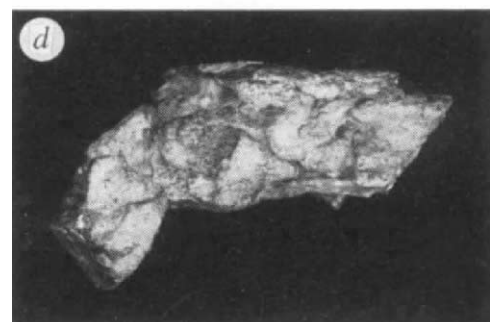
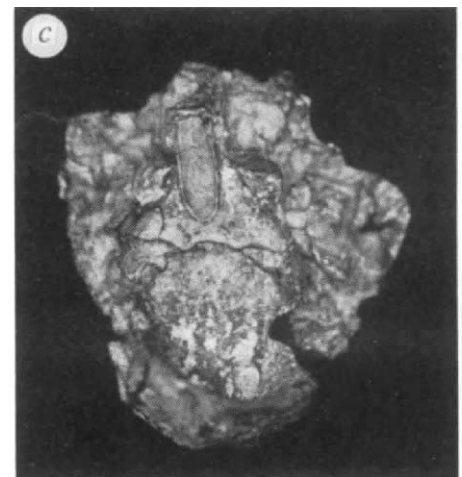
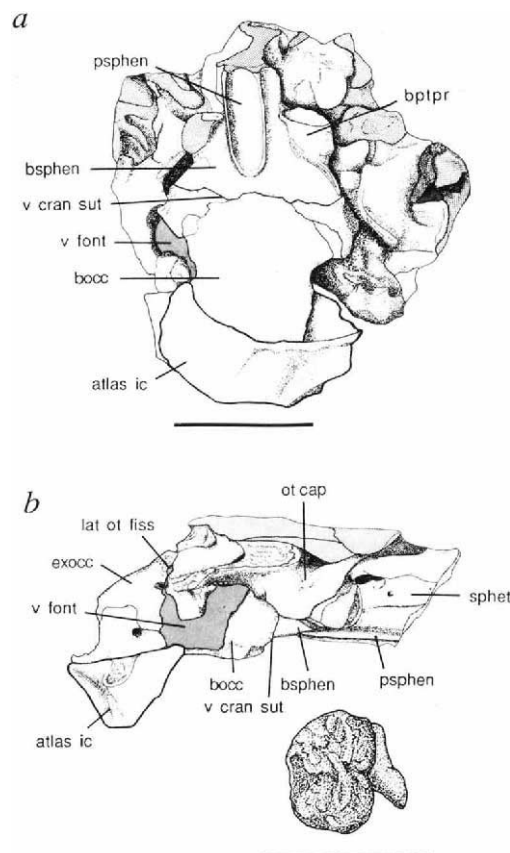
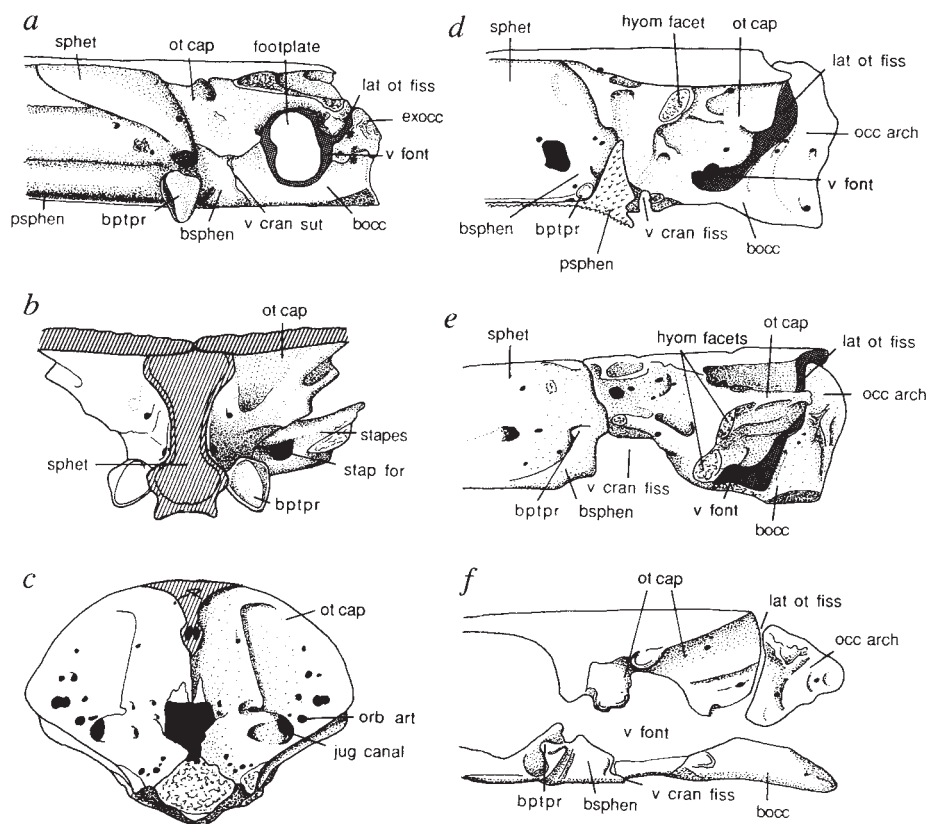




FIG. 2 Reconstructed braincases of fishes and *Acanthostega gunnari*. a, *A. gunnari*, lateral view, with stapedia footplate in vestibular fontanelle. b, *A. gunnari*, anterior view, as if sectioned through sphenethmoid, with left stapes indicated in position. c, *Mimia toombsi*, anterior view (from ref. 6). d, *Moythomasia durgaringa* lateral view (from ref. 6). e, *Eusthenopteron foordi*, lateral view (from ref. 18), restored cartilage in lateral otic fissure has been omitted; note that the vestibular fontanelle of *Eusthenopteron* shows the same relationships as those in the other braincases; all show the fundamental primitive gnathostome pattern. f, *Acanthodes bronni*, lateral view (from ref. 19). Note that though *Acanthodes* is a late-occurring acanthodian, its braincase structure is that expected of a primitive gnathostome. Sectioned bone, hatched; lateral otic fissure and vestibular fontanelle in a, d and e shown in mechanical stipple. Abbreviations as for Fig. 1.



ated by a groove in line with the stapedia foramen (Figs 1b inset and 2a). The head of the stapes bears a similar relationship to the jugular canal and orbital (stapedial) artery foramen that it would if it had replaced the lateral commissure of the fish braincase and those regions of the postorbital process which bear the facets for the hyomandibula<sup>9</sup> (Fig. 2b, c). If this suggestion is correct, several implications follow. First, the stapedia footplate in tetrapods incorporates both heads of the osteolepiform hyomandibula, which have joined mesially to incorporate a passage for the stapedia artery. The existence of the stapedia foramen is otherwise unexplained. The suggestion contrasts with the traditional interpretation in which the footplate equates only to the ventral head of the hyomandibula<sup>11,21</sup>. Second, the primitive condition for the stapedia footplate is to have a single head, corroborating what is actually known of primitive tetrapod stapes<sup>4</sup>. Thus, stapes possessing both two proximal heads and a stapedia foramen, such as those of early amniotes (for example *Captorhinus*) and some temnospondyls, show a derived condition.

This new information on the braincase and stapes from these specimens prompts an interpretation of stapedia function modified from that put forward in 1989<sup>4</sup>. It has become apparent that although the palate in many of the earliest tetrapods was only loosely incorporated into the skull root<sup>12</sup>, the stapes was firmly embedded into the braincase and formed the only point of contact between otic capsule and palate. *Acanthostega*, like other very early tetrapods, appears not to have been autostylic in the way so universally assumed<sup>22–24</sup> but still relied on the stapes to link braincase and palate<sup>9,12,25</sup>. One possible explanation for incorporation of the stapes into the braincase wall is that it may thereby have provided firmer support for the palate than an articulation onto the otic capsule as in the hyomandibula of fishes. A functional spiracle is still likely for *Acanthostega* and other early tetrapods, as suggested by the structure of the prootic wall and skull table, with muscles controlling the opening and closing of the spiracle inserting on the stapes as in *Polypterus*<sup>26</sup>. However, the stapes itself appears not to have been mobile as previously suggested. □

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- Coates, M. I. & Clack, J. A. *Nature* **352**, 234–236 (1991).
- Clack, J. A. & Coates, M. I. in *Deciphering the Natural World and the Role of Collections and Museums* (eds Hoch, E. & Brantsen, A. K.) 39–42 (Geological Museum Copenhagen, 1993).
- Coates, M. I. & Clack, J. A. *Nature* **347**, 66–69 (1990).
- Clack, J. A. *Nature* **342**, 425–430 (1989).
- Jarvik, E. *Basic Structure and Evolution of Vertebrates* Vol. 1 (Academic, London, 1980).
- Gardiner, B. G. *Bull. Br. Mus. (nat. Hist.) Geol.* **37**, 173–428 (1984).
- Gaffney, E. S. *Bull. Carn. Mus. nat. Hist.* **13**, 92–105 (1979).
- Panchen, A. L. & Smithson, T. R. *Biol. Rev.* **62**, 341–438 (1987).
- Smithson, T. R. & Thomson, K. S. *Zool. J. Linn. Soc.* **74**, 93–103 (1982).
- de Beer, G. R. *The Development of the Vertebrate Skull* (Clarendon, Oxford, 1937).
- Lombard, R. E. & Bolt, J. R. *Biol. J. Linn. Soc.* **11**, 19–76 (1979).
- Clack, J. A. in *The Evolutionary Biology of Hearing* (eds Webster, D. B., Fay, R. R. & Popper, A. N.) 405–420 (Springer, New York, 1992).
- Beaumont, E. I. *Phil. Trans. R. Soc. B* **280**, 29–101 (1977).
- Smithson, T. R. *Zool. J. Linn. Soc.* **76**, 29–90 (1982).
- Clack, J. A. & Holmes, R. B. *Palaeontology* **31**, 85–107 (1988).
- Romer, A. S. & Witter, R. V. *J. Geol.* **50**, 925–960 (1942).

- Panchen, A. L. & Smithson, T. R. in *The Phylogeny and Classification of Tetrapods*, Vol. 1: *Amphibians, Reptiles and Birds* (ed. Benton, M. J.) 1–32 (Clarendon, Oxford, 1988).
- Jarvik, E. *Kungl. svenska vetensk. acad. handl.* **5**, 1–104 (1954).
- Moy-Thomas, J. A. & Miles, R. S. *Palaeozoic Fishes* (Chapman and Hall, London, 1971).
- Vorobyeva, E. I. & Schultze, H.-P. in *Origins of the Higher Tetrapod Groups* (eds Trueb, L. & Schultze, H.-P.) 68–109 (Cornell Univ. Press, New York, 1991).
- Eaton, T. J. *J. Wash. Acad. Sci.* **29**, 109–117 (1939).
- Huxley, T. H. *Proc. zool. Soc. Lond.* **1876**, 24–59 (1876).
- Goodrich, E. S. *Studies on the Structure and Development of Vertebrates* 1958 edn (Dover, New York, 1930).
- Wake, M. H. *Hyman's Comparative Vertebrate Anatomy* 3rd edn (Univ. Chicago Press, Chicago, 1979).
- Carroll, R. L. in *The Terrestrial Environment and the Origin of Land Vertebrates* (ed. A. L. Panchen) 293–318 (Academic, London, 1980).
- Allis, E. P. *J. Anat.* **56**, 189–294 (1922).

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# The visual filter mediating letter identification

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**WE hear periodic sounds, or tones, by means of parallel auditory filters, each tuned to a band of temporal frequency<sup>1</sup>, and we see periodic patterns, or gratings, by means of parallel visual filters, each tuned to a band of spatial frequency<sup>2</sup>. Beyond helping us to see gratings, do these visual filters participate in everyday tasks such as reading and object recognition? After all, grating visibility only requires the distinguishing of pattern from blank, whereas object recognition, for example letter identification, requires classification by the observer into one of many learned categories. Here we make use of results from hearing research<sup>3</sup>, applying to vision a noise-masking paradigm that reveals the filter(s) mediating any threshold task. We find that letter-identification and grating-detection filters are identical, showing that the recognition of these objects at one size is mediated and constrained by a single visual filter, or 'channel'.**

Visual science has made great advances using tasks that de-emphasize cognitive processing in order to isolate 'low-level' physiological processes, such as the spatial frequency channels that mediate grating detection<sup>2,4</sup>. However, everyday visual tasks like reading<sup>5</sup> and object recognition<sup>6</sup> are cognitive, and it is unclear what role, if any, these channels play in such 'high-level' tasks. Letters, being over-learned, are good for testing the limits of object recognition. As letters—unlike gratings—are spatially compact and spectrally broad, we expected their identification to be mediated by multiple channels, or a much broader channel than the channel that mediates grating detection. Surprisingly, our noise-masking paradigm shows that the same channel per-

forms both low-level detection of narrow-band gratings and high-level identification of broad-band letters.

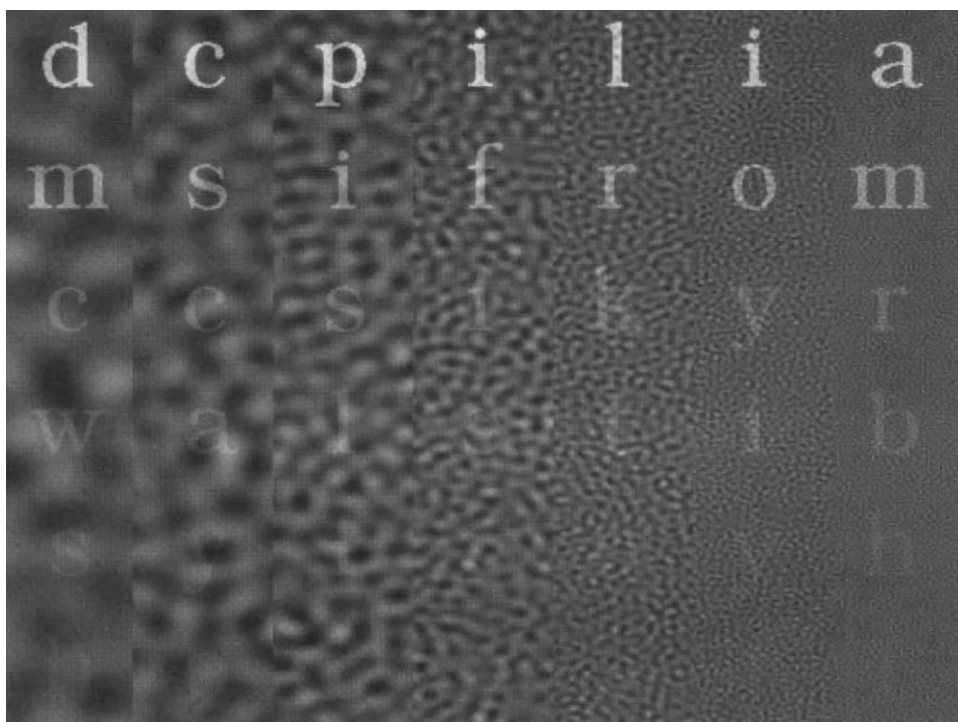
Figure 1 demonstrates the unexpected bandpass nature of letter identification. Noise at 3 cycles per letter masks letter identification more effectively than noise at lower or higher frequencies. The contrast sensitivity function traced out by the faintest identifiable letters has a bandwidth similar to that of filters for grating detection.

A first step towards understanding an object recognition process is to determine which data are used by the process. Figure 2 shows how the high- and low-pass noise-masking paradigm measures the tuning of the filter mediating any threshold task. We measure threshold contrast energy (that is, integrated squared contrast) for letter identification as a function of the cut-off frequency of the noise. Data from two observers are shown in Fig. 3.

We express these results in terms of a minimal channel model: a linear filter followed by a nonlinear decision. It has been demonstrated that threshold signal energy  $E$  is linearly related to the power spectral density of a white noise mask<sup>7,10</sup> but what about non-white noise? If we assume that  $E$  is linearly related to the total noise power passed by the linear filter, then the frequency dependence of the filter gain can be derived directly from either our high- or our low-pass masking results<sup>3,11,13</sup>. More generally, this total-filtered noise-power assumption allows one to derive the tuning of the observer's filter for any task as a function of any dimension, such as spatial frequency, orientation or space, directly from appropriate noise-masked threshold measurements. The derived filters for letter identification are shown in Fig. 4.

A remarkable feature of these results is that the filters estimated from high- and low-pass data are so similar. By analogy with off-frequency listening<sup>14</sup>, if an observer could choose one of many filters, then the ideal strategy would be to look off-frequency, choosing the filter that yields the best signal-to-noise ratio<sup>11</sup>. The observer has every incentive to avoid low-pass noise by choosing a high-frequency filter, and to avoid the high-pass noise by using a low-frequency filter. Yet the filters revealed by high- and low-pass masking are both centred at spatial frequen-

**FIG. 1** Letters in noise, demonstrating that, despite the broad  $1/f^2$  spectra of letters, noise at 3 cycles per letter (middle column) masks letter identification more effectively than noise at lower or higher frequencies. Read down each column as far as you can; the positions of the faintest identifiable letters trace out the sensitivity of your eye's letter-identification channel as a function of spatial frequency. Note that changes in viewing distance, from 3 to 60 cm, hardly affect the visibility of any given letter, indicating that the channel scales with letter size. (Identification of letters in noise is independent of size for letters subtending 0.5 to 15.8 degrees of visual angle<sup>22</sup>.) Letter contrast (luminance increment divided by background luminance) decreases by factors of  $\times 0.588$  from 0.89 in the top row to 0.063 in the bottom row. The noise contrast  $c_{rms}$  is 0.22. Centre frequency (cycles per letter) of the noise's octave-wide pass band is: 0.8, d, m, c, w, s, h; 1.3, c, s, c, a, y, o; 2.0, p, i, s, l, f, t; 3.2, i, f, i, s, r, w; 5.1, l, r, k, t, o, s; 8.1, i, o, y, i, y, f; 13, a, m, r, b, h, t.





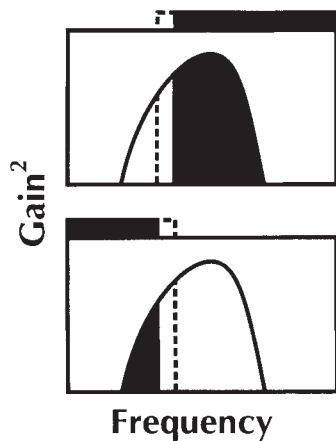


FIG. 2 The high- and low-pass noise masking paradigm. The curves represent the power gain (that is, gain squared) of the observer's hypothetical filter. The bar along the top of each graph represents the noise spectrum. Increasing the bandwidth of the noise adds noise in a new band (outlined by dashes). The effect of the noise in that band can be gauged by the resultant change in threshold, and independent estimates are provided by the high- and low-pass experiments. If we assume that the threshold energy is proportional to total filtered noise power, which is the black area under the filter's power gain curve, then the change in threshold specifies the filter's gain in the new band.

cies of  $\sim 3$  cycles per letter, indicating that higher- and lower-frequency filters must be far less efficient or incapable of mediating letter identification.

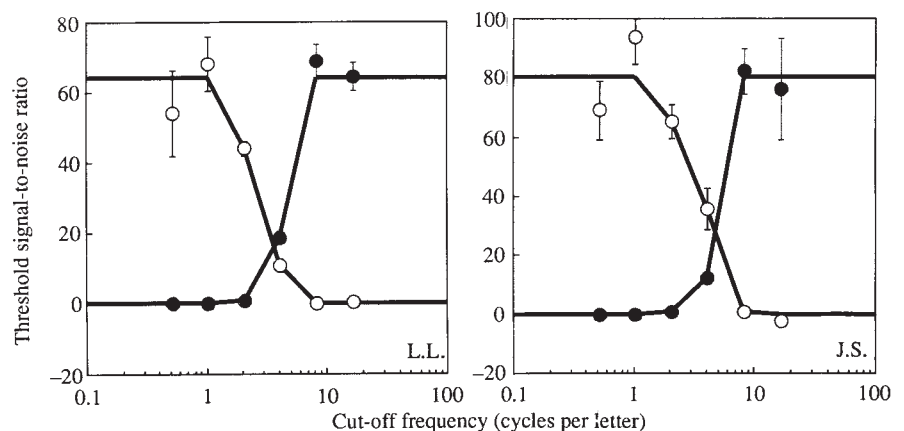
The bandpass nature of these filters is characteristic of spatial frequency channels, whose one-to-two octave-wide tuning functions (at half height) have classically been estimated by grating summation<sup>15</sup>, masking<sup>12,16,18</sup> and identification at threshold<sup>19</sup>. For a direct comparison, we applied our high- and low-pass noise-masking paradigm to a grating identification task, measuring the filter used by an observer to identify the orientation of a grating that was tilted right or left by  $45^\circ$ . This task is known to produce thresholds similar to those obtained in simple detection experiments<sup>20</sup>. Although the  $\sim 1/f^2$  letter spectrum is utterly different from the narrow-grating spectrum, Fig. 4c and d shows that letter and grating identification are mediated by very similar filters, and off-frequency looking is no greater with the broad-band letters than it is with narrow-band gratings. The simplest explanation of this result is that one channel mediates and constrains object recognition; that is, the filter reflects the architecture of the visual system.

Of course, at least in principle, the (broad) average letter spectrum is irrelevant to letter identification; what matters is the differences between letters, and no single spectrum can summarize the many pairwise differences. Might the letter-derived filter

merely reflect the demands of the letter-identification task, rather than a constraint of human vision? To determine what filter would reflect the task, we implemented the ideal classifier for letters in white noise, and applied our masking paradigm to it. The ideal classifier maximizes the probability of a correct response by choosing the hypothesis (letter identity) with maximum a posteriori probability. The white-noise ideal classifier chooses the letter that minimizes total squared difference between letter and stimulus, normalized by twice the noise variance, minus the letter's log prior probability<sup>21</sup>. The white-noise ideal classifier received the same information, as numbers, that was presented to the human observers as visual stimuli. The white-noise ideal classifier is ideal only when the noise is white.

The ratio of the ideal's threshold energy to that of a particular observer defines the observer's efficiency at that task. Observer L.L.'s and J.S.'s efficiencies at high levels of white noise, when  $E^* \gg E_0$ , are 13% and 10% for letter identification, and 6% and 8% for grating identification. Higher efficiencies have been reported: up to 42% for letter identification<sup>22</sup> and up to 70% for grating discrimination<sup>7</sup>. However, in the former case, stimuli were band-pass-filtered, which may boost efficiency<sup>23</sup>, and in the latter, the task was discrimination of near-threshold contrasts of otherwise identical gratings, which increases efficiency as much as 9-fold over detection<sup>24,25</sup>.

FIG. 3 Threshold signal-to-noise ratio,  $E^*/N$ , for letter identification as a function of cut-off frequency of high pass (open symbols) or low pass (filled symbols) noise, where  $N$  is the spectral density of the noise mask and  $E^* = E - E_0$  denotes the difference in threshold contrast energy for identification between noise-masked and non-masked letters. Letter size is specified by the font's x height, which was  $0.95^\circ$ . As rendered in the Adobe Bookman font, 14 of the 26 lower-case letters in the English alphabet—acemnoruvwxz—have the same height (top to bottom) as an x, and the rest—bfghijklpqty—are higher. When occurring at English language frequency (that is, many e and few j characters)<sup>28</sup>, as in our experiments, the average letter height and width are 1.13 and 1.08 x heights, respectively. Letters were presented randomly, for 195 ms, at their English language frequencies. As shown in Fig. 2, the noise, which was static and isotropic, had power spectral densities of zero and  $N$  on either side of the cut-off frequency. All stimuli were displayed with gamma correction<sup>29</sup> on a monochrome CRT with a background luminance of  $71 \text{ cd m}^{-2}$ . The VideoToolbox software<sup>29</sup> used in these experiments is available by anonymous ftp of 'info-mac/dev/src/video-toolbox...' from sumex-aim.stanford.edu. The viewing distance was 120 cm. The



spatial extent of each noise mask was  $3.9^\circ \times 3.9^\circ$ . Each plotted symbol represents a maximum likelihood estimate of threshold (63% correct) based on at least 4 sessions of 30 trials each<sup>30</sup>. Standard error of each threshold measurement is indicated. Subsequent analysis of each observer's thresholds used maximum-likelihood, monotonic fits (solid curves) constrained to be positive with equal white-noise asymptotes.

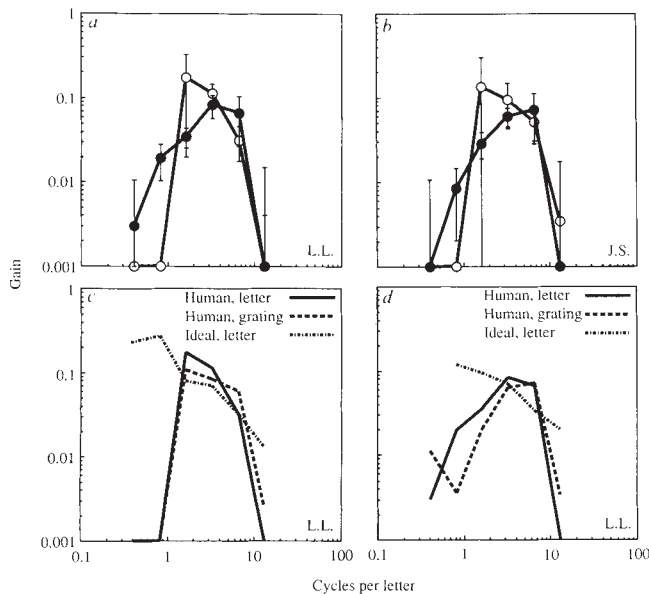


FIG. 4 *a* and *b*, Letter-identification filters for two subjects L.L. and J.S., as derived from Fig. 3. Error bars contain the 68% confidence interval about each plotted gain, as determined below. Zero gain is indicated by plotting the symbol on the horizontal axis. Filters plotted in *a* are replotted in *c* (high pass) and *d* (low pass) for comparison with similarly derived filters for grating identification by the same observer and letter identification by the white-noise ideal. (The other observer, J.S., produced similar results, which are not shown.) Each sinusoidal luminance grating had a spatial frequency of 3 cycles per letter, or 3.2 cycles per degree, tilted 45° to the left or right, and its contrast was windowed by a spatial gaussian envelope with a space constant of one grating period, centred midway between a peak and trough. Filter derivation: We assume that the threshold  $E$  is linearly related to the total noise power passed by some filter  $G(f, \theta)$ ,

$$E = E_0 + a \int_0^\infty df \int_0^{2\pi} d\theta G^2(f, \theta) N(f, \theta) \quad (1)$$

where  $E_0$  is the threshold measured without display noise,  $a$  is a proportionality constant,  $f$  is radial spatial frequency,  $\theta$  is orientation,  $G^2(f, \theta)$

Figure 4*c* and *d* shows that the white-noise-ideal-derived filter—which is a pure reflection of the demands of the task—is low-pass, unlike the human-derived filters, which are bandpass. The high gain of the ideal filter at low frequencies indicates the presence of information relevant to letter identification at these frequencies. The rapid fall-off of the gain of the human filters at frequencies below 1.5 and above 6 cycles per letter indicates that the human identifying letters is insensitive outside this two-octave band. The high-frequency fall-off parallels the finding that adding noise between 10 and 40 cycles per picture height is

is the filter's power gain, and  $N(f, \theta)$  is the power spectral density of the displayed noise. (This is equivalent to assuming a constant signal-to-noise ratio at the output of the filter at threshold.)  $E_0$  may be due to intrinsic visual noise<sup>8</sup>; we subtract it from all of our data, leaving just the threshold elevation  $E^* = E - E_0$  due to the displayed noise. The low- and high-pass noise spectra are

$$N_{\text{low}}(f, \theta) = \begin{cases} N & \text{for } f < f_c \\ 0 & \text{for } f \geq f_c \end{cases}$$

$$N_{\text{high}}(f, \theta) = \begin{cases} 0 & \text{for } f < f_c \\ N & \text{for } f \geq f_c \end{cases}$$

where  $f_c$  is the cut-off frequency. Substituting each of these noise spectra into equation (1), differentiating with respect to cut-off frequency, and solving for the filter's average power gain across all orientations yields

$$G^2(f) = \frac{1}{2\pi a f N} \frac{dE_{\text{low}}^*}{df} \quad (2)$$

and

$$G^2(f) = \frac{-1}{2\pi a f N} \frac{dE_{\text{high}}^*}{df} \quad (3)$$

where  $E_{\text{low}}^*$  and  $E_{\text{high}}^*$  are the threshold elevations produced by low- and high-pass noise, as a function of cut-off frequency. Comparisons among filters are facilitated by normalizing the gain so that all the filters pass equal power from a white noise input. This is achieved by setting the equation (1) proportionality constant to  $a = E_{\text{all}}^*/N$ , where  $E_{\text{all}}^*$  is the threshold elevation in white 'all pass' noise with power spectral density  $N$ . In our experiments we only used discrete values of cut-off frequency,  $f_i, f_{i+1}, \dots$ , so we use discrete approximations to equations (2) and (3),

$$G^2\left(\sqrt{\frac{f_{i+1}^2 + f_i^2}{2}}\right) \approx \frac{1}{\pi E_{\text{all}}^*} \frac{E_{\text{low}}^*(f_{i+1}) - E_{\text{low}}^*(f_i)}{f_{i+1}^2 - f_i^2}$$

and

$$G^2\left(\sqrt{\frac{f_{i+1}^2 + f_i^2}{2}}\right) \approx \frac{-1}{\pi E_{\text{all}}^*} \frac{E_{\text{high}}^*(f_{i+1}) - E_{\text{high}}^*(f_i)}{f_{i+1}^2 - f_i^2}$$

which are exact if  $G^2(f)$  is constant over the interval  $f_i$  to  $f_{i+1}$ .

much more effective in suppressing face recognition than adding noise between 40 and 70 cycles per picture height<sup>26</sup>. The low-frequency fall-off confirms the human observers' inability to identify severely low-passed noisy letters, which are still identifiable by an ideal observer<sup>22</sup>, and severely impaired character legibility<sup>27</sup> and reading rate<sup>5</sup> for text optically low-pass filtered to less than 2 cycles per character width. Thus the channel mediating letter identification is very different from what one would expect solely from a consideration of the task, and instead represents a visual constraint upon object recognition. □

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1. Fletcher, H. *Rev. Mod. Phys.* **12**, 47–65 (1940).
2. Campbell, F. W. & Robson, J. G. *J. Physiol.* **197**, 551–556 (1968).
3. Patterson, R. D. *J. Acoust. Soc. Am.* **55**, 802–809 (1974).
4. Graham, N. V. S. *Visual Pattern Analyzers* (Oxford University Press, Oxford, 1989).
5. Legge, G. E., Pelli, D. G., Rubin, G. S. & Schleske, M. M. *Vision Res.* **25**, 239–252 (1985).
6. Farah, M. J. *Visual Agnosia: Disorders of Object Recognition and What They Tell Us About Normal Vision* (MIT Press, Cambridge, MA, 1990).
7. Burgess, A. E., Wagner, R. F., Jennings, R. J. & Barlow, H. B. *Science* **214**, 93–94 (1981).
8. Pelli, D. G. thesis, Univ. Cambridge (1981).
9. Pelli, D. G. in *Vision: Coding and Efficiency* (ed. Blakemore, C.) 3–24 (Cambridge University Press, 1990).
10. Pavel, M., Sperling, G., Riedl, T. & Vanderbeek, A. *J. Opt. Soc. Am.* **A4**, 2355–2365 (1987).
11. Pelli, D. G. *Invest. Ophthalmol. Vis. Sci.* suppl. **19**, 44A (1980).
12. Henning, G. B., Hertz, B. G. & Hinton, J. L. *J. Opt. Soc. Am.* **71**, 574–581 (1981).
13. Pelli, D. G. & Watson, A. B. *Perception* **12**, A6–A7 (1983).
14. Patterson, R. D. & Nimmo-Smith, I. *J. Acoust. Soc. Am.* **67**, 229–245 (1980).
15. Graham, N. & Robson, J. G. *Vision Res.* **27**, 1997–2007 (1987).
16. Pantle, A. & Sekuler, R. *Science* **162**, 1146–1148 (1968).
17. Stromeyer, C. F. & Julesz, B. *J. Opt. Soc. Am.* **62**, 1221–1232 (1972).

18. Wilson, H. R., McFarlane, D. K. & Phillips, G. C. *Vis. Res.* **23**, 873–882 (1983).
19. Watson, A. B. & Robson, J. G. *Vis. Res.* **21**, 1115–1122 (1981).
20. Thomas, J. P. & Gille, J. J. *Opt. Soc. Am.* **69**, 652–660 (1979).
21. Van Trees, H. L. *Detection, Estimation and Modulation Theory* (Wiley, New York, 1968).
22. Parish, D. H. & Sperling, G. *Vision Res.* **31**, 1399–1416 (1991).
23. Pelli, D. G., Jacknowski, M. M. & Hoepner, J. A. *Noninvasive Assessment of the Visual System, 1990 Tech. Digest Ser.* **3**, 144–146 (Opt. Soc. Am., Washington DC, 1990).
24. Nachmias, J. & Sansbury, R. V. *Vis. Res.* **14**, 1039–1042 (1974).
25. Barlow, H. B. *J. Physiol.* **160**, 155–168 (1962).
26. Harmon, L. D. & Julesz, B. *Science* **180**, 1194–1197 (1973).
27. Ginsburg, A. P. *Proc. of the Society for Information Display* **21**, 219–227 (1980).
28. Kucera, H. & Francis, W. N. *Computational Analysis of Present-Day American English* (Brown University Press, Providence, RI, 1967).
29. Pelli, D. G. & Zhang, L. *Vis. Res.* **31**, 1337–1350 (1991).
30. Watson, A. B. & Pelli, D. G. *Percept. Psychophys.* **33**, 113–120 (1983).

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# ***atonal* is the proneural gene for *Drosophila* photoreceptors**

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THE *Drosophila* peripheral nervous system comprises four major types of sensory element: external sense organs (such as mechanosensory bristles), chordotonal organs (internal stretch receptors), multiple dendritic neurons, and photoreceptors. During development, the selection of neural precursors for external sense organs requires the proneural genes of the *achaete-scute* complex, which encode basic-helix-loop-helix transcription factors<sup>1-3</sup>. These genes do not, however, control precursor selection for chordotonal organs or photoreceptors<sup>4,5</sup>, raising the question of whether other proneural genes exist<sup>6</sup> or a different mechanism of neurogenesis operates. Here we show that *atonal* (*ato*), originally isolated as a proneural gene for chordotonal organs<sup>7</sup>, is also the proneural gene for photoreceptors. Pattern formation in the *Drosophila* eye involves a succession of cell fate specifications. Of the eight photoreceptors within each ommatidium of the compound eye, the photoreceptor R8 is the first to appear in the eye imaginal disc, right behind the morphogenetic furrow<sup>8-10</sup>. The appearance of other photoreceptors (R1-7) follows in a defined sequence that is thought to arise by induction from R8 (refs 8, 9, 11, 12). We find that photoreceptor formation requires the function of *atonal* at the morphogenetic furrow and that *atonal* is specifically required for R8 selection. Formation of other photoreceptors does not directly require *atonal* function, but does depend on R8 selection by *atonal*. Thus, photoreceptors are selected by two mechanisms: R8 by a proneural mechanism, and R1-7 by local recruitment.

A mutant of *ato* (*ato*<sup>1</sup>) is semi-lethal and behaves as a genetic null. Surviving adults lack chordotonal organs (manuscript in preparation). In addition, *ato*<sup>1</sup> mutant flies (either homozygotes or hemizygotes (heterozygotes over a deletion of the gene)) are almost eyeless, and also lack ocelli (Figs 1*a, b* and 2*a, b*). All that remains externally of the compound eye is a small red patch with a smooth surface, which is studded with a few interommatidial bristles. The red patch clearly represents pigment cells and is affected by mutations of *white* (*w*). This phenotype represents an extreme version of those mutations that result in reduced ommatidial numbers, such as *Ellipse* mutants, although these always have ommatidia remaining among the pigment cells<sup>13</sup>. Head sections of mutant adults reveal the complete absence of photoreceptors and reduction in size of the optic lobe (Fig. 1*c, d*). Ommatidial loss can be partially rescued by generalized expression of *ato* in transgenic flies (Fig. 2).

Examination of eye imaginal discs from third instar larvae shows that there is a failure of photoreceptor selection in the mutant. In the wild-type eye disc, photoreceptor selection begins at the morphogenetic furrow as it traverses the ectoderm of the disc from posterior to anterior; eventually, ommatidial clusters of eight photoreceptors (R1-8) can be detected behind the furrow by antibodies such as the monoclonal antibody 22C10 (ref. 14) (Fig. 3*a*). In *ato* mutant discs, no photoreceptors are detectable with this antibody (Fig. 3*b*), neither are early markers of neural precursors, including *neuralized* and *deadpan* (results not shown). The *scabrous* (*sca*) product, which is normally expressed during the selection of the first photoreceptor<sup>15</sup>, R8, is also absent from mutant discs (Fig. 3*c, d*). These results indicate that *ato* is required for the earliest steps of photoreceptor formation. In the mutant eye disc there is a shallow crease in the posterior that appears to be a remnant morphogenetic furrow (Fig. 3*a, b*; and our unpublished results). Thus, while movement of the morphogenetic furrow depends on photoreceptor

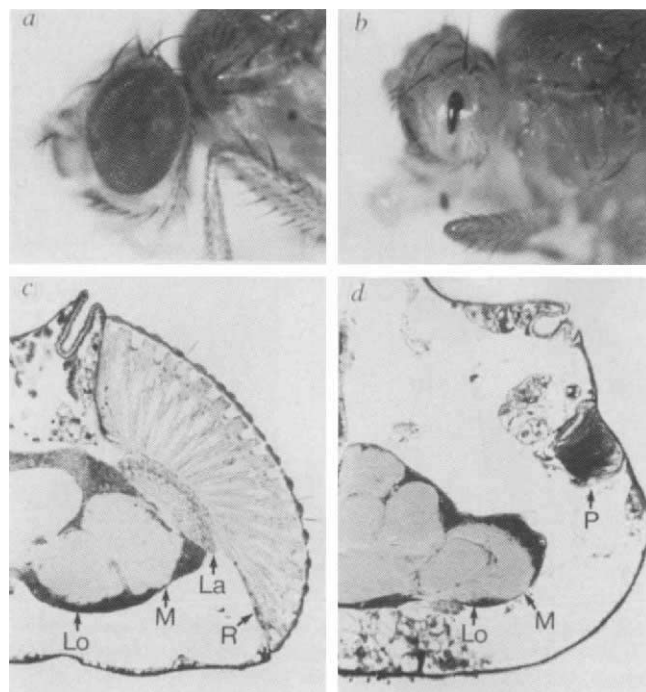


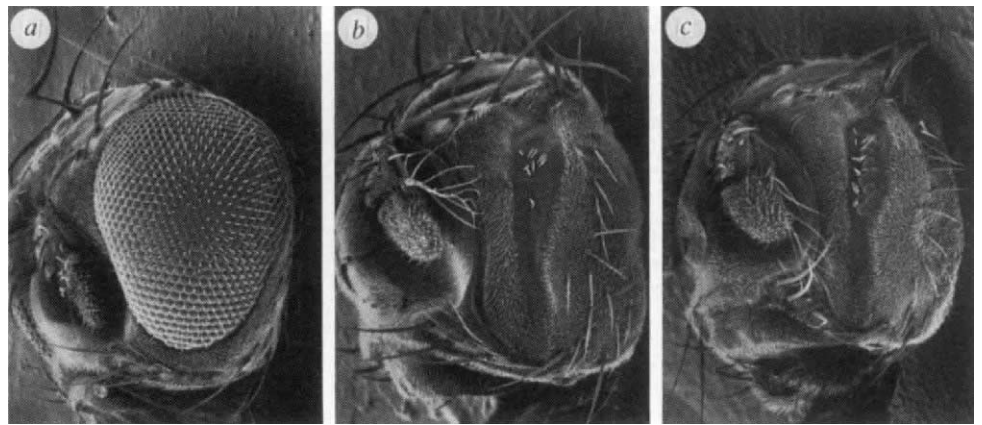
FIG. 1 Eye phenotype of *ato*<sup>1</sup>. *a*, Wild type. Each of the ~800 ommatidia in the compound eye lattice contains eight photoreceptors. *b*, *ato* mutant. The eye is reduced to a stripe of pigment cells. This fly contains the *ato*<sup>1</sup> mutation over a deletion of the region, *Df(3R)p*<sup>13</sup>. *ato*<sup>1</sup> homozygotes have an identical phenotype, but rarely eclose, probably because of other lesions on the chromosome. *c, d*, Horizontal head sections stained with toluidine blue. *c*, Wild type, showing the ommatidia of the retina (R), and the three layers of the optic lobe: La, lamina; M, medulla; Lo, lobula and lobula plate. *d*, *ato*<sup>1</sup>/*Df(3R)p*<sup>13</sup>. Photoreceptors are absent, the eye is reduced to a mass of pigment cells (P). The lamina is also absent, and the medulla is strongly reduced. This is probably a secondary effect of retinal loss<sup>22,23</sup>, although we note that *ato* is expressed in part of the optic lobe anlagen (unpublished results). METHODS. Ethyl methanesulphonate-induced mutations were first selected for lethality over *Df(3R)p*<sup>13</sup>, which uncovers the chromosomal region to which *ato* has been localized<sup>7</sup>, and then tested for lethality over *Df(3R)dsx29* and viability over *Df(3R)D7* (refs 7, 24). Mutations meeting all these criteria were analysed for chordotonal organ defects. Of 30 lethal or semi-lethal chromosomes over *Df(3R)p*<sup>13</sup>, one semi-lethal showed a specific absence of embryonic chordotonal neurons when stained with antibody 22C10 (*ato*<sup>1</sup>). We could detect no difference in the extent of chordotonal neuron loss in homozygous *ato*<sup>1</sup> embryos from that previously reported for deficiencies of the region<sup>7</sup>; thus *ato*<sup>1</sup> behaves as a genetic null. We have isolated several additional *ato* alleles with a similar phenotype to *ato*<sup>1</sup>. The *ato*<sup>1</sup> protein is predicted to have three amino-acid substitutions compared with the sequence from the isogenized chromosome used for the mutagenesis (sequenced in parallel). One of these (N261→I) occurs in a motif of the basic domain (NARER) that is conserved between ATO, Achaete-Scute, and Daughterless proteins. The second change (K253→N) occurs a short distance proximally, on the edge of the basic domain. The third change (A25→T) is near the N terminus.

formation behind the furrow<sup>16,17</sup>, some characteristics of the morphogenetic furrow appear to remain in the *ato* mutant. Posterior to the crease, the disc is strongly atrophied (Fig. 3*a, b*) as a result of massive apoptosis (not shown).

The photoreceptor R8 is the first of the eight photoreceptors within each ommatidium to appear, and it is thought that the others accrue by a succession of local inductions emanating from R8 (refs 8, 9, 11, 12). To determine experimentally which photoreceptors require autonomous *ato* function, we generated X-ray-induced somatic clones of homozygous *ato*<sup>1</sup> cells in developing eye discs of heterozygous larvae. Homozygous *ato*<sup>1</sup> tissue,

FIG. 2 Rescue of ommatidia by *ato* expression. Scanning electron micrographs of heads. *a*, Wild type; *b*, *ato<sup>1</sup>/Df(3R)p<sup>13</sup>*. The smooth pigment cell patch is dotted with bristles (many of which appear to be broken off). *c*, *ato<sup>1</sup>/Df(3R)p<sup>13</sup>* fly containing P-element constructs that allow generalized *ato* expression upon exposure to heat shock<sup>7</sup>. This fly had been subjected to a 15-min heat-shock pulse during larval development. A row of ommatidia is formed within the pigment cell patch. Ommatidia are of variable size, perhaps indicating that they contain variable numbers of photoreceptors. The spatially restricted rescue of ommatidia suggests that only a stripe of cells can respond to *ato* at a given time. Longer or multiple heat shocks result in pupal death.

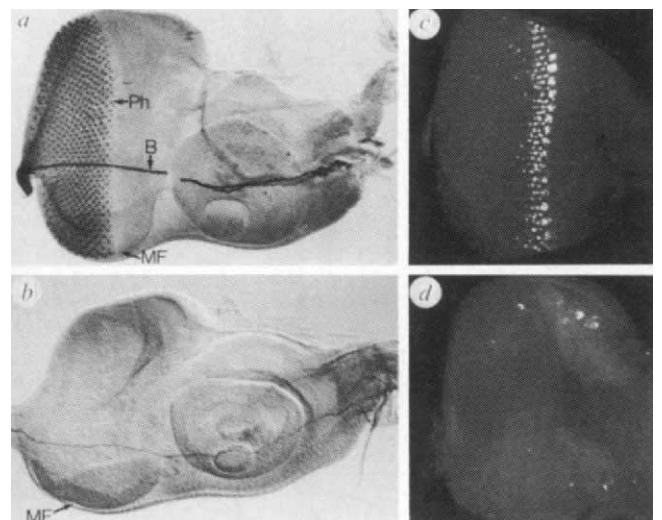
METHODS. *ato* induction was achieved using the GAL4 system<sup>25</sup> with the construct, stocks and conditions described previously<sup>7</sup>. The two elements were both insertions on the second chromosome, and a recombinant chromosome was used for experiments; that is, the genotype for the mutant was *w*; P[*w<sup>+</sup>*, *hs-GAL4*] P[*w<sup>+</sup>*, UAS-*ato*]/+; *ato<sup>1</sup>/*



*Df(3R)p<sup>13</sup>*. For micrography, adult flies were anaesthetized with ether. Their heads were removed in 0.1 M sodium phosphate (pH 7.2) with 0.3% Triton X-100, and fixed overnight in 2% glutaraldehyde and 4% formaldehyde in sodium phosphate. The heads were dehydrated in ethanol and critical-point dried in CO<sub>2</sub>. After mounting, specimens were sputtered with palladium-gold and photographed.

FIG. 3 Phenotype in *ato<sup>1</sup>* eye-antennal discs from third instar larvae. *a*, *b*, Discs stained with antibody 22C10 to detect sensory neurons. *a*, Wild type. Photoreceptor clusters (Ph) are detected posterior to (left of) the morphogenetic furrow (MF), which spans the disc from dorsal to ventral. The nerve of the larval photoreceptors (Bolwig nerve, B) traverses the disc. *b*, *ato<sup>1</sup>/Df(3R)p<sup>13</sup>*. Photoreceptors are absent and the Bolwig nerve is strongly reduced. A crease that may correspond to the morphogenetic furrow is still present, and the disc is strongly atrophied posterior to this. Blebbing in this region indicates extensive cell death. *c*, *d*, Expression of Sca protein detected by confocal microscopy. *c*, Wild type. Sca is a secreted protein that is expressed in small clusters of cells within the morphogenetic furrow and subsequently becomes refined to the R8 precursors<sup>15</sup>. Sca expression is thought to be part of the process of lateral inhibition within the furrow that results in the regular spacing of R8 cells<sup>10</sup>. *d*, *ato<sup>1</sup>/Df(3R)p<sup>13</sup>*. Sca is not detectable, indicating that no selection of R8 cells is occurring.

METHODS. Immunohistochemistry has been described<sup>7</sup>. For Sca staining, a mouse antibody was used (X. Hu and N. Baker, unpublished results), and discs were fixed in PLP<sup>8</sup>.



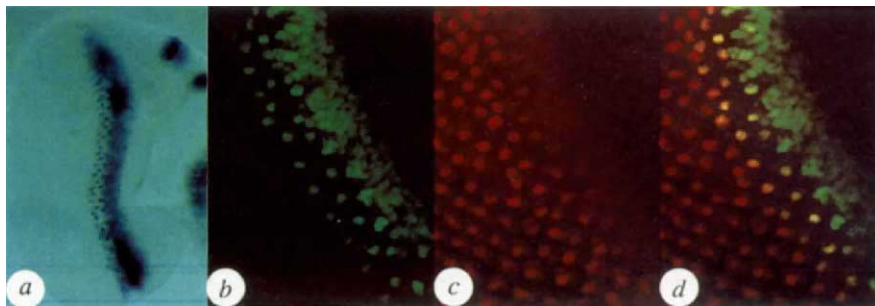
marked by the loss of a *w<sup>+</sup>* gene on the wild-type chromosome, results in a scar due to absent ommatidia, but this is surrounded by mostly normally constructed ommatidia containing mixtures of wild-type and *ato* photoreceptors. Scoring for *w<sup>+</sup>* (and therefore *ato<sup>1</sup>*) photoreceptors in correctly assembled mosaic ommatidia, we find that any photoreceptor can be mutant except R8 (fractions of mutant (*w<sup>-</sup>*) photoreceptors in 61 mosaic ommatidia: R1, 0.59; R2, 0.31; R3, 0.49; R4, 0.43; R5, 0.23; R6, 0.52; R7, 0.66; R8, 0). Thus, only R8 absolutely requires endogenous *ato* function for correct formation. R8 must then recruit R1–7 from the surrounding ectoderm in a process that does not absolutely require proneural gene function. Therefore, the absence of R1–7 (and accessory cells) in the *ato<sup>1</sup>* eye is a consequence of *ato* requirement to form R8. Our data substantiate previous indications that R8 may play a central role in ommatidial assembly<sup>10,18,20</sup>. Given the variation in frequencies between photoreceptors R1–7, we cannot rule out the possibility that some photoreceptors other than R8 may prefer to be *ato<sup>+</sup>* (particularly R2 and 5). For instance, the transient *ato* expression in the furrow (see below) may predispose cells surrounding R8 to receive later inductive signals.

The expression of *ato* is consistent with the mosaic analysis. For chordotonal organs, we previously showed that *ato* mRNA is expressed in ectodermal proneural clusters and later in the chordotonal organ precursors that arise from these clusters<sup>7</sup>. In the eye disc, *ato* messenger RNA is expressed in a stripe spanning the disc on the anterior edge of the morphogenetic furrow (Fig. 4a), thereby defining it as a proneural cluster for photoreceptors. As the furrow passes, this expression becomes refined, presumably by lateral inhibition<sup>10,19</sup>, to isolated, regularly spaced cells. To identify these isolated cells, we used an enhancer trap line of the *Star* (*S*) locus<sup>18</sup>, which expresses *lacZ* strongly in R8, and weakly in R2 and 5. Double-labelling experiments with antibodies against Ato and  $\beta$ -galactosidase show that the isolated Ato-expressing cells are the newly formed precursors of R8 only (Fig. 4b–d).

It may seem surprising that chordotonal organs and photoreceptors should require the same proneural gene. Structurally, chordotonal organs have more in common with external sense organs than with photoreceptors, and organs of intermediate structure are known from other insects<sup>21</sup>. Yet, unlike most external sense organs, chordotonal organs and photoreceptors both



FIG. 4 *ato* is expressed anterior to the morphogenetic furrow and is refined to R8 only. *a*, *ato* mRNA is expressed strongly on the anterior edge of the furrow. This becomes refined initially to clusters of cells, then to isolated cells about 2–3 rows (corresponding to 4–6 h of development) beyond (left of) the furrow (marked by the arrowhead). In this view, expression is also seen in two small patches of ectoderm from which the ocelli precursors arise (upper right), and on the edge of the proneural cluster for the antennal chordotonal organs (extreme right). *b–d*, Confocal microscope images of eye discs double-labelled with anti-Ato (green) and anti- $\beta$ -galactosidase (red) antibodies, from a line with a P-element enhancer trap insertion at *Star*<sup>18</sup>. *b*, Ato is expressed in a pattern similar to the mRNA. *c*, In this line,  $\beta$ -galactosidase is expressed strongly in R8 after its formation beyond the furrow, and weakly in R2 and R5 flanking each R8. *d*, Superimposed image: Ato and  $\beta$ -galactosidase are coexpressed (yellow) in the initial rows of R8 precursors.



METHODS. RNA detection was by whole-mount *in situ* hybridization using a digoxigenin-labelled *ato* probe. For protein double labellings, a rabbit anti-Ato serum (raised against bacterially expressed Ato fusion protein, detailed description and characterization in preparation) and mouse anti- $\beta$ -galactosidase (Promega) were used.

exist as organized, closely apposed clusters of neurons. In the eye, the basis for this clustering is neural induction, with only the founding R8 photoreceptor arising from the *ato* proneural cluster. For embryonic chordotonal organs, there are also too few *ato*-expressing precursors to account for the final number of neurons in each chordotonal array, implying that there are also founding and recruited chordotonal precursors (in preparation). It is tempting to suggest, therefore, that *ato*-derived neural precursors differ from precursors derived from the *achaete-scute* complex in that they can recruit further precursors by induction.

In conclusion, *ato* and the *achaete-scute* complex can account for the origin of almost the entire peripheral nervous system. The *achaete-scute* complex has provided the paradigm of a two-step genesis of neural precursors: proneural genes provide neural competence; neurogenic genes restrict this<sup>1–3</sup>. As a mechanism for forming arrays of similar neurons, *ato* may invoke a further step to this process: induction of secondary precursors by the first wave of founding precursors. An interesting possibility is that the recruited precursors may only indirectly require proneural gene function. In the vertebrate nervous system, there is a frequent requirement for aggregations of like neurons. Similar processes may be involved. □

## Implications for *bcd* mRNA localization from spatial distribution of *exu* protein in *Drosophila* oogenesis

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SUBCELLULAR RNA localization in different cell types leads to asymmetric distribution of proteins in these cells<sup>1,2</sup>. The localization of *bicoid* (*bcd*) messenger RNA to the anterior pole of the developing *Drosophila* oocyte gives rise in embryogenesis to a steep concentration gradient of the *bcd* protein<sup>3–6</sup>, a transcription factor that activates expression of zygotic genes needed for anterior development<sup>7–9</sup>. The *exuperantia* (*exu*) gene is necessary for this localization of *bcd* mRNA<sup>3,4</sup>. Here we express a chimaeric gene encoding a fusion between the *Acquorea victoria* green fluorescent protein (GFP)<sup>10</sup> and the *exu* protein (Exu) in female germ cells, and find that the fusion protein fluoresces strongly in both live and fixed cells during *Drosophila* oogenesis. The fusion protein rescues an *exu* null allele, restoring full fertility to females, and is expressed and localized in a temporal and spatial pattern similar to native Exu. The high sensitivity of the GFP tag provides important new details on the subcellular localization of Exu. The fusion protein is found in particles concentrated at ring canals, where transport occurs between nurse cells and the oocyte. Drugs such as colchicine and taxol that affect microtubule stability alter localization of the particles. We propose that the particles are ribonucleoprotein complexes or vesicles which transport *bcd* mRNA along microtubules and target it to the anterior oocyte cortex.

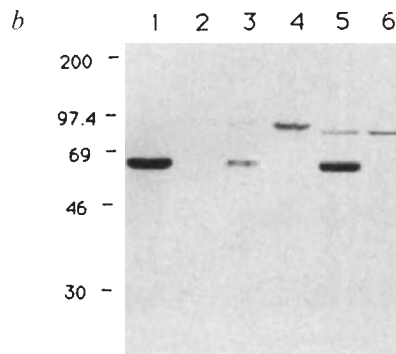
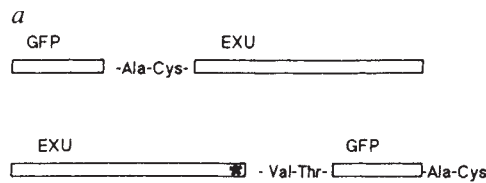
Because of the essential roles of the *exu* gene<sup>3,4</sup> and of microtubules<sup>11</sup> in *bcd* mRNA localization, we investigated whether Exu interacts with the microtubule cytoskeleton during oogenesis. To study the subcellular distribution of Exu in oogenesis, we constructed chimaeric genes (Fig. 1*a*) to encode N- and C-terminal fusions of Exu to GFP. These constructs were transformed into flies by P-element-mediated transformation (Fig. 1 legend). Both fusion proteins were expressed in oogenesis, and fluoresced strongly when excited with blue light. Ovaries from

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1. Ghysen, A. & Dambly-Chaudière, C. *Trends Genet.* **56**, 251–255 (1989).
2. Campuzano, S. & Modolelli, J. *Trends Genet.* **8**, 202–208 (1992).
3. Ghysen, A., Dambly-Chaudière, C., Jan, L. Y. & Jan, Y. N. *Genes Dev.* **7**, 723–733 (1993).
4. Dambly-Chaudière, C. & Ghysen, A. *Genes Dev.* **1**, 297–306 (1987).
5. Jiménez, F. & Campos-Ortega, J. A. *J. Neurogen.* **4**, 179 (1987).
6. Basler, K. & Hafen, E. *BioEssays* **13**, 621–631 (1991).
7. Jarman, A. P., Grau, Y., Jan, L. Y. & Jan, Y. N. *Cell* **73**, 1307–1321 (1993).
8. Tomlinson, A. & Ready, D. F. *Dev Biol.* **120**, 366–376 (1987).
9. Ready, D. F. *Trends Neurosci.* **12**, 102–110 (1989).
10. Baker, N. E., Mlodzik, M. & Rubin, G. M. *Science* **250**, 1370–1377 (1990).
11. Banerjee, U. & Zipursky, S. L. *Neuron* **4**, 177–187 (1990).
12. Rubin, G. R. *Trends Genet.* **7**, 372–377 (1991).
13. Baker, N. E. & Rubin, G. M. *Nature* **340**, 150–153 (1989).
14. Zipursky, S. L., Venkatesh, T. R., Teplow, D. B. & Benzer, S. *Cell* **36**, 15–26 (1984).
15. Mlodzik, M., Baker, N. E. & Rubin, G. M. *Genes Dev.* **4**, 1848–1861 (1990).
16. Heberlein, U., Wolff, T. & Rubin, G. M. *Cell* **75**, 913–926 (1993).
17. Ma, C., Zhou, Y., Beachy, P. A. & Moses, K. *Cell* **75**, 927–938 (1993).
18. Heberlein, U., Hariharan, I. K. & Rubin, G. M. *Dev Biol.* **160**, 51–63 (1993).
19. Cagan, R. L. & Ready, D. L. *Genes Dev.* **3**, 1099–1112 (1989).
20. Karpiow, J., Kolodkin, A., Bork, T. & Venkatesh, T. *Genes Dev.* **3**, 1834–1844 (1989).
21. McIver, S. B. in *Comprehensive Insect Physiology, Biochemistry and Pharmacology* Vol. 6 (eds Gilbert, L. I. & Kerkut, D. A.) (Pergamon, New York, 1985).
22. Meyerowitz, E. M. & Kankel, D. R. *Dev Biol.* **62**, 112–142 (1978).
23. Fischbach, K. F. & Technau, G. *Dev Biol.* **104**, 219–239 (1984).
24. Lindsley, D. L. & Zimm, G. G. in *The Genome of Drosophila melanogaster* (Academic, San Diego, 1992).
25. Brand, A. H. & Perrimon, N. *Development* **118**, 401–415 (1993).

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**FIG. 1** Construction and transformation of *gfp-exu* fusion genes. **a**, Fusion proteins expressed from *gfp-exu* chimaeras. Amino acids in addition to those in GFP or Exu (boxes) are shown. **b**, Flies express only the fusion GFP-Exu protein from the chimaeric genes, and no detectable cleavage products. Ovary extracts were prepared from *exu*<sup>+</sup> flies (lanes 1, 3, 5) and from *exu*<sup>Sco2</sup> (*exu* null) flies (lanes 2, 4, 6) carrying an N-terminal *gfp-exu* fusion gene inserted on the third chromosome (P[Cas,NGE]3; lanes 3, 4), or a C-terminal *exu-gfp* fusion gene inserted on the X chromosome (P[Cas, CGE]1; lanes 5, 6). *M*<sub>s</sub> of endogenous Exu and the GFP-Exu fusion protein are 60K and 86K, respectively (*M*<sub>s</sub> of standards ( $\times 10^{-3}$ ) are shown on the left).

**METHODS.** The Muta-Gene *in vitro* mutagenesis kit (Bio-Rad) was used to introduce *Bst*II and *Sph*I sites immediately before the translation initiation codon or immediately before the TAA stop codon of the *exu* gene in a 5.5-Kb *Sma*I-*Eco*RI genomic fragment. The GFP coding region from cDNA pGFP10.1 (refs 10, 21) was amplified by PCR, using oligonucleotides that placed *Bst*II and *Sph*I cloning sites at the 5' and 3' ends, respectively (oligonucleotides from Operon Technologies, CA), and cloned into either the N terminus or the C terminus of the mutated *exu* gene. In the case of the C-terminal fusion, the last amino acid of Exu, Asn (asterisk in **a**), was changed to Lys to create the *Bst*II site. Sequences of the chimaeric genes were checked at their fusion sites<sup>22</sup> and subcloned into the *Drosophila* transformation vector, p[CaSpeR4]<sup>23</sup>, generating P[Cas,NGE] and P[Cas,CGE]. A *y w*<sup>67c23</sup> fly strain was transformed using the helper plasmid *p* $\pi$ 25.7wc<sup>24,25</sup>. Ten independent transformants were obtained with p[Cas,NGE] and five with p[Cas,CGE]. Two independent insertions of each construct were introduced into an *exu*<sup>Sco2</sup> mutant background, and each rescued the maternal effect lethal phenotype. A rat polyclonal anti-Exu antibody was used to detect the presence of Exu and/or GFP-Exu in ovary protein extracts (**b**) as previously described<sup>13</sup>.

transformed females, homozygous for an *exu* null allele, expressed only the full-length fusion protein (Fig. 1b, lanes 4 and 6), and both fusion proteins rescued the maternal effect lethal phenotype of this *exu* null allele.

As both fusion proteins are identical in terms of function and subcellular localization, both will be referred to as 'GFP-Exu'. GFP-Exu fluoresces in living cells and in fixed specimens (Fig. 2) in a pattern that confirms previous observations on *exu* expression during oogenesis<sup>12,13</sup>. However, the GFP tag gave

greater sensitivity and resolution than standard antibody staining techniques, allowing us to extend our previous results. The GFP tag has several advantages: (1) GFP was resistant to photobleaching; (2) the amplification of signal that results from the binding of multiple antibody molecules to a single antigen molecule is offset by the <100% efficiency of fluorochrome incorporation onto the antibody and of binding of the antibodies to their targets. When the transformed chimaeric genes were introduced into an *exu* null background, every molecule of Exu

**FIG. 2** Localization of GFP-Exu in live and fixed *Drosophila* egg chambers. Confocal optical sections of developing egg chambers (anterior is to the left). **a**, Fixed germarium and previtellogenic egg chambers. GFP-Exu (green) accumulates in the oocyte (arrows) during stages 1–7. The larger arrow points to the oocyte in a stage-1 egg chamber. **b**, Live previtellogenic stages showing localization to the oocyte. Localization of GFP-Exu to subcellular particles is apparent by stage 7 (egg chamber on the right) and persists through later stages. By stage 7 there is also patchy localization around nurse cell nuclei. **c**, Fixed stage-6 (left) and -9 (right) egg chambers. The anterior (A) and posterior (P) ends of the stage-9 oocyte are indicated by arrows. In stage-9 oocytes, GFP-Exu forms a broad anterior concentration gradient, and is also localized to the posterior pole. In nurse cells, a ring of GFP-Exu has developed around the cell periphery. **d**, Live stage-10 egg chamber; arrows indicate the anterior (A) and posterior (P) ends of the oocyte. GFP-Exu is still localized to the posterior of the oocyte, and at the anterior is more tightly localized than in stages 8–9. A faint ring of cortical GFP-Exu is also present in the oocyte. **e**, Background fluorescence in a fixed *wild-type Canton S* stage-10 egg chamber lacking GFP-Exu.

**METHODS.** For observations of live egg chambers, ovaries were dissected from 2–3-day-old females in modified Robb's medium<sup>17</sup>. Egg chambers were observed with a Bio-Rad MRC600 3-colour laser confocal microscope with a filter providing 488 and 568 nm excitation and 522  $\pm$  17 nm emission wavelengths. Ovaries were fixed in formaldehyde<sup>17</sup> for 10 min, then washed 3 times with PBS, and mounted in 50% glycerol in PBS. As noted in ref. 10, some fingernail polishes used to seal the edges of coverslips decrease the fluorescence of GFP. The genotype for egg chambers in **a–d** was *cn exu*<sup>Sco2</sup> *bw/cn exu*<sup>Sco2</sup> *bw*; P[Cas,NGE]3/+.

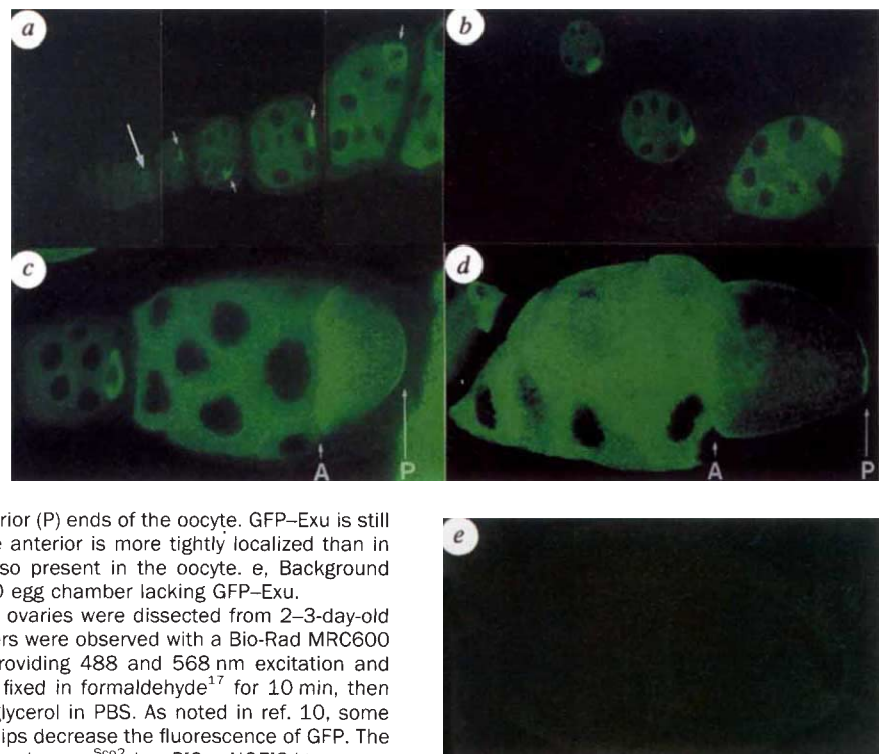
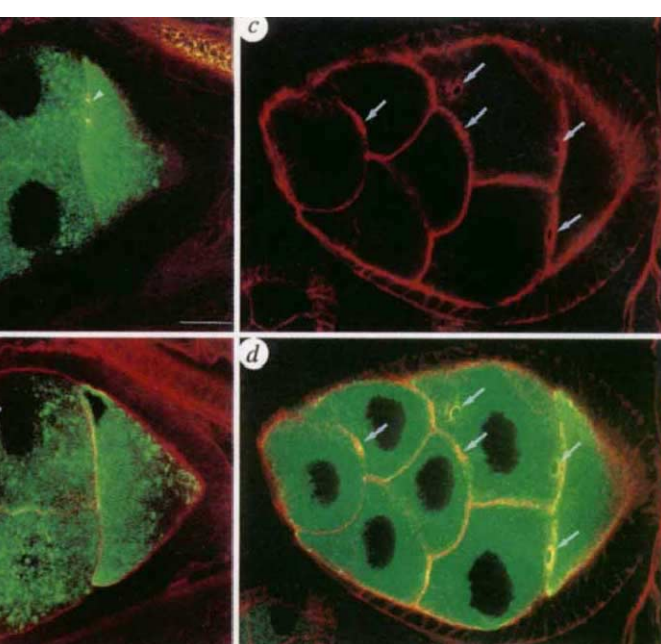




FIG. 3 GFP-Exu is present in particles that appear to be transported through the ring canals connecting the nurse cells and the oocyte. *a* and *b*, Confocal optical sections of an early stage-9 egg chamber showing the localization of GFP-Exu (green) and rhodamine-conjugated phalloidin (red) which reveals the positions of the actin-rich ring canals; the oocyte is on the right in all panels. Particles containing GFP-Exu are concentrated near ring canals (arrows). The section shown in *a* is through the middle of a ring canal connecting a nurse cell to the oocyte (arrowhead). In this case, GFP-Exu appears to be passing through the ring canal. *c* and *d*, Early stage-9 egg chamber treated with colchicine *in vitro* for 10 min; *c* shows the rhodamine signal alone to indicate the positions of the ring canals (arrows) and *d* shows both GFP-Exu and rhodamine-phalloidin. The concentration of GFP-Exu particles at ring canals (arrows) is greatly reduced. The larger particles containing GFP-Exu are less abundant, and small particles appear more uniformly distributed in the cytoplasm. Rhodamine-phalloidin staining also reveals the presence of a ring of actin around the periphery of each nurse cell and the oocyte, as observed previously<sup>15</sup>. This staining is variable in both control and colchicine-treated egg chambers, and is not altered systematically by colchicine treatment. The *exu* genotype of the females was *exu<sup>Sco2</sup>/exu<sup>Sco2</sup>; P[Cas,NGE]3/+*.

**METHODS.** Colchicine (Sigma) was dissolved in 100% ethanol (20 mg ml<sup>-1</sup>). Ovaries from 2–3-day-old females were dissected in Schneider's medium with 10% bovine fetal calf serum (BRL) and incubated in 50 µg ml<sup>-1</sup> colchicine in the same medium. All drug treatments were done in glass wells in a moisturized chamber with gentle shaking



at room temperature. Control ovaries were incubated with the addition of 0.25% ethanol. Ovaries were fixed (Fig. 2 legend) after 10, 20, 30 and 60 min of treatment and stained for F-actin with 5 units ml<sup>-1</sup> rhodamine-phalloidin (Molecular Probes) in PBS buffer for 15 min, followed by 3 washes with PBS. Scanning for GFP and rhodamine signals was done separately and the two images were then merged, using the ENHANCE program supplied with Bio-Rad software.

was tagged; (3) background signals caused by nonspecific binding of primary and secondary antibodies decrease resolution.

As with the endogenous *exu* gene, the chimaeric gene is expressed in females only in the germ-cell-derived nurse cells and oocyte, and not in the somatic follicle cells (Fig. 2). With GFP-Exu we confirmed the early localization to the oocyte in previtellogenic egg chambers (Fig. 2*a, b*), its anterior concentration in stage-8–10 oocytes (Fig. 2*c, d*), and its presence in perinuclear patches in nurse cells (Fig. 2*b*). GFP-Exu also shows the same temporal lability as Exu, being absent in mature oocytes. Back-

ground fluorescence in control egg chambers lacking GFP-Exu is minimal (Fig. 2*e*).

This technique has revealed important new details in the Exu pattern of subcellular localization. The oocyte nucleus in stage-8 egg chambers is embedded in a dense matrix of GFP-Exu (not shown). A faint ring of GFP-Exu is detected around the periphery of nurse cells (Fig. 2*c*), and a faint ring of subcortical GFP-Exu is also present in stage-10 oocytes (Fig. 2*d*). Some GFP-Exu is localized to the posterior pole of oocytes during stages 8–10*a* (Fig. 2*c, d*). We therefore re-examined wild-type egg chambers by whole-mount antibody staining, which con-

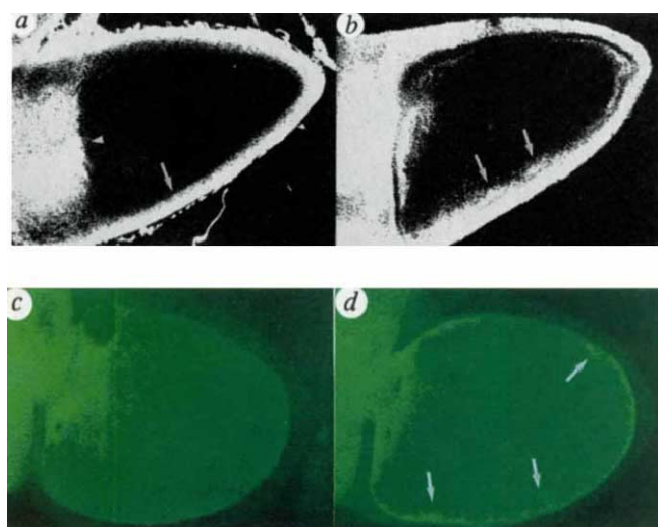


FIG. 4 Taxol induces microtubules and localized GFP-Exu at similar ectopic sites. *a*, Microtubules in a Canton S (wild-type) stage-10 oocyte, visualized by antibody staining. Anterior, left; posterior, right. The bright

staining around the periphery of the egg chamber is due to microtubules present in the follicle cells which surround the oocyte. Microtubules are more concentrated at the anterior pole (arrowhead) and a faint ring of staining is apparent around the oocyte cortex (arrow). *b*, Microtubules in a Canton S stage-10 oocyte treated with taxol. Patches of ectopic microtubules are present near the oocyte lateral cortex (arrows). *c*, GFP-Exu in an untreated stage-10 oocyte. GFP-Exu is present at higher concentrations at the anterior of the oocyte, in a faint peripheral ring around the oocyte, and some is localized to the posterior pole. *d*, GFP-Exu in a taxol-treated stage-10 oocyte. GFP-Exu is present in higher concentrations at the lateral oocyte cortex, including patches that extend into the interior of the oocyte (arrows). The *exu* genotype of the females for *c* and *d* was *exu<sup>Sco2</sup>/exu<sup>Sco2</sup>; P[Cas,NGE]3/+*.

**METHODS.** Taxol (2 mM) was dissolved in dimethyl sulphoxide. Drug treatment was as described in Fig. 3 legend, except that incubation with taxol was for 2 h. Taxol was administered at 20 µM, and control egg chambers were incubated in 1% DMSO. For observations of microtubules, whole-mount antibody staining was done as described<sup>19</sup>, with monoclonal anti- $\alpha$ -tubulin primary antibody (Sigma) at a 1:300 dilution, and goat anti-mouse IgG TRITC (tetramethylrhodamine isothiocyanate) conjugate (Sigma) secondary antibody (preabsorbed against fixed *Drosophila* ovaries) at a 1:100 dilution. As a control for nonspecific binding by secondary antibody, some egg chambers were stained with secondary antibody only, which gave some rhodamine signal in follicle cells but no signal in the oocyte. Egg chambers were mounted in PBS with 50% glycerol.

firmed that Exu protein was posteriorly localized (data not shown). The *exu* gene may provide a function in posterior segmentation, as 6% of embryos from *exu* mutant mothers have defects in abdominal segmentation<sup>14</sup>.

The most important new information provided by GFP-Exu is that the protein is localized to particles that are most abundant in stage-8–10 egg chambers (Figs 2, 3 and 4). We have previously noted a 'punctate' appearance of antibody-stained Exu in sectioned egg chambers<sup>13</sup>, consistent with the existence of these particles. Staining of egg chambers with rhodamine-conjugated phalloidin reveals the actin-rich ring canals<sup>15</sup> (Fig. 3) through which factors synthesized in nurse cells are transported between nurse cells and from the nurse cells into the developing oocyte<sup>16</sup>. The particles containing GFP-Exu are concentrated in nurse cells near ring canals and appear in the centre of ring canals connecting the nurse cells to the oocyte (Fig. 3a, b).

The subcellular localization of GFP-Exu resembles the distribution of microtubules in both nurse cells and oocytes in *Drosophila* oogenesis<sup>11,17–19</sup>. To test for a GFP-Exu/microtubule association, we investigated the effects of colchicine and taxol, drugs that have opposite effects on microtubule stability, on the subcellular distribution of GFP-Exu. When egg chambers were treated with colchicine in culture medium, particles no longer accumulated around ring canals and they became more uniformly distributed in the cytoplasm within 10 min (Fig. 3d). In untreated control egg chambers, the particles varied greatly in size (Fig. 3a, b). After colchicine treatment, larger particles were less abundant (Fig. 3d). These results indicate that the large particles may result from the aggregation of small particles by a process that is affected by microtubules. The microtubule-stabilizing agent taxol causes the formation of ectopic microtubule aggregates and also ectopically localizes *bcd* mRNA in an *exu*-dependent fashion in stage-10 oocytes<sup>11</sup>. We have confirmed that taxol induces ectopic microtubules at lateral sites near the oocyte cortex (Fig. 4b) and find that GFP-Exu is also ectopically localized to these same regions (Fig. 4d).

We propose that the particles visualized with GFP-Exu are ribonucleoprotein (RNP) complexes or vesicles which contain *bcd* mRNA and are targeted to the anterior oocyte cortex by their attachment to microtubules. Alternatively, the particles might associate with another subcellular structure which is itself localized by microtubules. We do not know whether the role of Exu is to form the particles or to mediate their subcellular localization, but we do not believe that Exu is involved in direct binding to *bcd* mRNA. We have found that although Exu binds RNA *in vitro*, this binding is nonspecific and is mediated through two basic regions of the protein which are not required for localization of *bcd* RNA *in vivo* (S.W. and T.H., unpublished results). It is likely that another protein present in these particles binds directly and specifically to *bcd* mRNA. Localization of mRNA as microtubule-associated particles may be a general phenomenon. Myelin basic protein mRNA, injected into cultured neurons, associates with particles that move along the dendrites and are possibly attached to microtubule tracts<sup>20</sup>. The association of GFP-Exu with particles should provide a useful biochemical handle for their purification and characterization. □

15. Warn, R. M., Gutzeit, H. O., Smith, L. & Warn, A. *Exp. Cell Res.* **157**, 355–363 (1986).
16. Spradling, A. C. *The Development of Drosophila melanogaster* 1–70 (Cold Spring Harbor Press, New York, 1993).
17. Theurkauf, W. E., Smiley, S., Wong, M. L. & Alberts, B. M. *Development* **115**, 923–936 (1992).
18. Gutzeit, H. *Wilhelm Roux Arch. dev. Biol.* **195**, 173–181 (1986).
19. Theurkauf, W. E., Alberts, B. M., Jan, Y. N. & Jongens, T. A. *Development* **118**, 1169–1180 (1993).
20. Ainger, K. et al. *J. Cell Biol.* **123**, 431–441 (1993).
21. Prasher, D. C., Eckenrode, V. K., Ward, W. W., Prendergast, F. G. & Cormier, M. J. *Gene* **111**, 229–233 (1992).
22. Sanger, F., Brownlee, G. G. & Barrell, B. G. *Proc. natn. Acad. Sci. U.S.A.* **71**, 5463–5467 (1977).
23. Thummel, C. & Pirrotta, V. *Drosophila Information Service* **71**, 150 (1992).
24. Rubin, G. & Spradling, A. *Science* **218**, 348–353 (1982).
25. Karsenti, R. & Rubin, G. *Cell* **38**, 135–146 (1984).

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## Cytotoxic T-cell activity antagonized by naturally occurring HIV-1 Gag variants

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Most asymptomatic individuals infected with HIV-1 have a cytotoxic T lymphocyte (CTL) response to the virus Gag proteins which can be demonstrated *in vitro*<sup>1,2</sup>. Epitopes have been mapped in p17 Gag and p24 Gag restricted by HLA-B8 (p17-3 and p24-13) and -B27 (p24-14)<sup>2,3</sup>. Viruses isolated from patients who make CTL responses to these peptides vary within the genetic sequences encoding these epitopes and some mutations lead to reduction in killing activity *in vitro*<sup>4</sup>. This was attributed to either failure of the variant epitope to bind major histocompatibility complex class I or failure of T-cell receptors to bind the presented peptide. But peptide variants of class I-restricted epitopes cause 'antagonism', that is, the presence of a variant epitope (in the form of peptide) inhibits normal lysis of targets presenting the original epitope<sup>5,6</sup>. This mirrors similar findings in class II-restricted systems<sup>7–10</sup>. Here we report that naturally occurring variant forms of p17-3, p24-13 and p24-14 may cause antagonism of CTL lines derived from the same individuals. The effect is present if the epitopes are derived from synthetic peptides and when they are processed from full-length proteins expressed by either recombinant vaccinia constructs or replicating HIV.

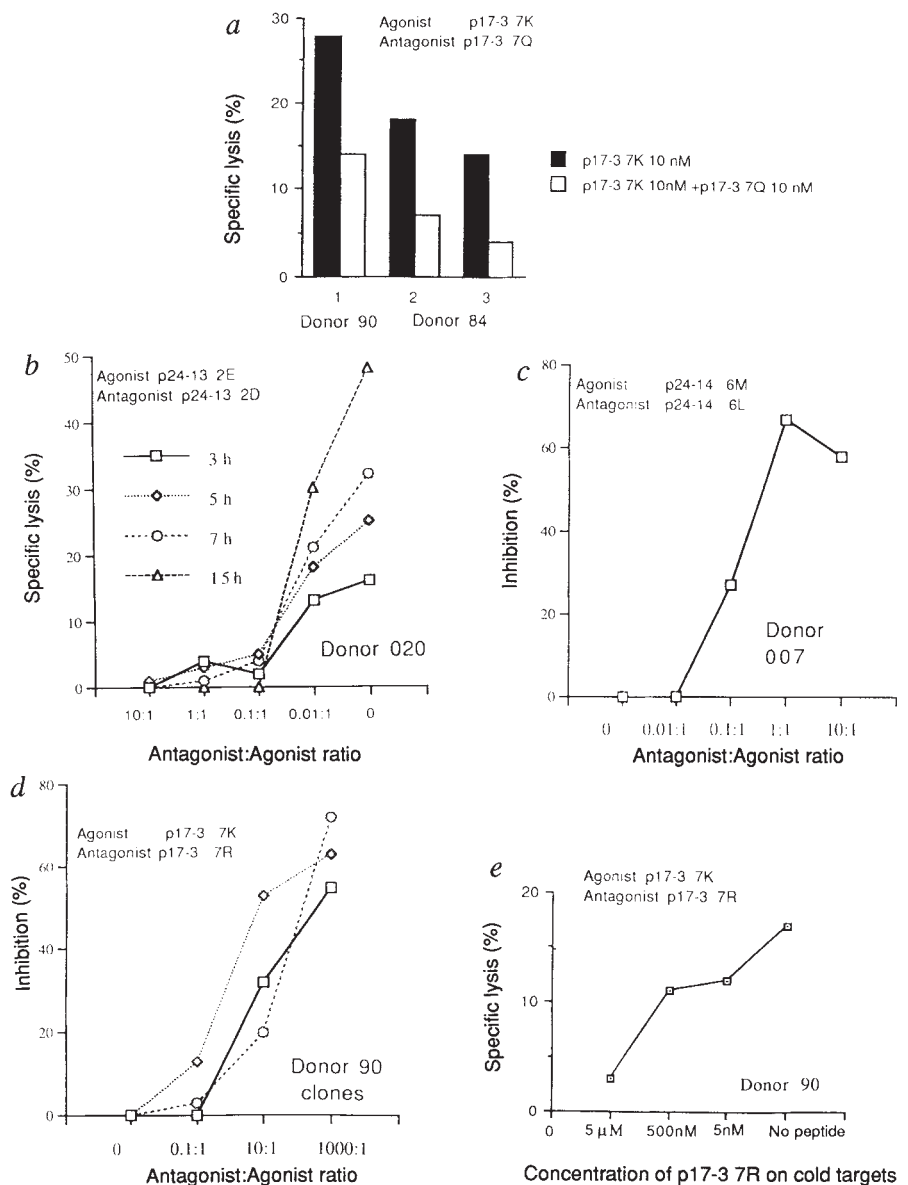
Variation in HIV-1 Gag epitopes that affects CTL recognition has been previously reported<sup>4</sup>. To investigate this and its relationship to CTL antagonism further, a number of CTL lines were generated from infected individuals, specific for either p17-3, p24-13 or p24-14 (Table 1). Sequence data on p17 and p24 Gag were obtained from proviral DNA from the same donors to determine variation present within these epitopes. In addition, variant p17-3 sequences were detected in plasma RNA in donors 84 and 90. Synthetic peptides representing these variant epitopes were then tested in T-cell receptor (TCR) antagonist assays using the protocol of Jameson *et al.*<sup>5</sup>. In addition, p17-3-specific clones

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1. Singer, R. H. *Curr. Biol.* **4**, 15–19 (1992).
2. Wilhelm, J. E. & Vale, R. D. *J. Cell Biol.* **123**, 269–274 (1993).
3. Berlieth, T. et al. *EMBO J.* **7**, 1749–1756 (1988).
4. Johnston, D., Driever, W., Berlieth, T., Richstein, S. & Nusslein-Volhard, C. *Development* **107** suppl., 13–19 (1989).
5. Frigerio, G., Burri, M., Bopp, D., Baumgartner, S. & Noll, M. *Cell* **47**, 735–746 (1986).
6. Driever, W. & Nusslein-Volhard, C. *Cell* **54**, 83–93 (1988).
7. Schroder, C., Tautz, D., Seifert, E. & Jackle, H. *EMBO J.* **7**, 2881–2887 (1988).
8. Struhl, G., Struhl, K. & Macdonald, P. M. *Cell* **57**, 1259–1273 (1989).
9. Driever, W., Thomas, G. & Nusslein-Volhard, C. *Nature* **340**, 363–367 (1989).
10. Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W. & Prasher, D. C. *Science* **263**, 802–805 (1994).
11. Pokrywka, N. J. & Stephenson, E. C. *Development* **113**, 55–66 (1991).
12. Macdonald, P. M., Luk, S. K. & Kilpatrick, M. *Genes Dev.* **5**, 2455–2466 (1991).
13. Marcey, D., Watkins, W. S. & Hazelrigg, T. *EMBO J.* **10**, 4259–4266 (1991).
14. Schüpbach, T. & Wieschaus, E. *Wilhelm Roux Arch. dev. Biol.* **195**, 302–317 (1986).



**FIG. 1** Effective CTL antagonism by naturally occurring variants in epitopes p17-3, p24-13 and p24-14. CTL lines and sublines were derived from donors and maintained as described previously<sup>2,17</sup>. The line from donor 007 was stimulated using variant peptide 6M, to which a predominant response had been obtained. Targets were in all cases autologous or HLA matched Epstein-Barr virus transformed B cell lines. In screening assays to test for agonist activity, <sup>51</sup>Cr-labelled targets were prepulsed with 50  $\mu$ M p17-3 7K (GGKKKYKLK) or 7R (GGKKKYRLK), p24-13 2E (GEIYKRWII) or p24-14 6M (KRWIIMGLNK) (see Table 1). Peptides were synthesized using standard Fmoc techniques, purity determined by HPLC and concentrations determined using the BCA system. Targets were incubated in round-bottomed wells at 37 °C with  $5 \times 10^3$  or  $10^4$  targets per well and either effector cells at E:T ratio 4:1 for clones 10:1 for lines, medium or Triton X in a total volume of 150  $\mu$ l. Supernatant was assayed at 4–6 h and specific lysis (SL) determined as  $100 \times (\text{experimental lysis} - \text{spontaneous lysis}) / (\text{maximum lysis} - \text{spontaneous lysis})$ . To investigate antagonism, labelled targets were prepulsed with a suboptimal concentration of agonist peptide (p17-3 7K = 10 nM, p24-13 2E = 1  $\mu$ M and p24-14 6M = 1  $\mu$ g ml<sup>-1</sup>) and identical assays done in the presence of antagonist peptide at the ratios shown. Inhibition was determined at  $100 \times (\text{SL without antagonist} - \text{SL with antagonist}) / \text{SL without antagonist}$ . **a**, Inhibition of lysis of p17-3 7K pulsed cells (10 nM) by p17-3 7Q variant (see Table 1) at antagonist:agonist ratio of 1:1. Effectors were: 1, donor 90 line E:T ratio 10:1; 2, 3, donor 84 lines E:T 10:1 and 5:1, respectively. Lysis of cells without peptide (0–2%) in each case subtracted. **b**, Inhibition of lysis of p24-13 2E pulsed cells (1  $\mu$ M) by p24-13 2D variant at antagonist:agonist ratio 0.1:1–10:1. Effectors were a p24-13 2E-specific cell line derived from donor 020 at E:T ratio 10:1. This effect was demonstrable for 3–15 h of assay. **c**, Inhibition of lysis of p24-14 6M pulsed cells (1  $\mu$ g ml<sup>-1</sup>) by p24-14 6L at antagonist:agonist ratio 0.1:1–10:1. Effectors were a p24-14 6M-specific cell line derived from donor 007 at E:T ratio 10:1. Specific lysis in the absence of antagonist was 33%. **d**, Inhibition of lysis of p17-3 7K-pulsed cells (5 nM) by p17-3 7R at antagonist:agonist ratio 0.1:1–1,000:1. Effectors were three donor 90-derived p17-3-specific clones E:T ratio 4:1. Specific lysis in the absence of antagonist was 31%, 43% and 37% for clones 5 (squares), 9 (circles) and 15 (diamonds). TCRs for these clones were V $\alpha$  15.1, 3.1 and 4.1, V $\beta$  7.1, 9.1 and 6.3, respectively. **e**, Inhibition of lysis of labelled (hot) p17-3 7K-pulsed cells (5 nM) by p17-3 7R variant pulsed on to unlabelled (cold) targets. Cold:hot ratio was 5:1. Effectors were pre-incubated with cold targets for 3 h before addition of labelled cells. Effectors were donor 90 subline 20, E:T ratio 4:1.



tively. **e**, Inhibition of lysis of labelled (hot) p17-3 7K-pulsed cells (5 nM) by p17-3 7R variant pulsed on to unlabelled (cold) targets. Cold:hot ratio was 5:1. Effectors were pre-incubated with cold targets for 3 h before addition of labelled cells. Effectors were donor 90 subline 20, E:T ratio 4:1.

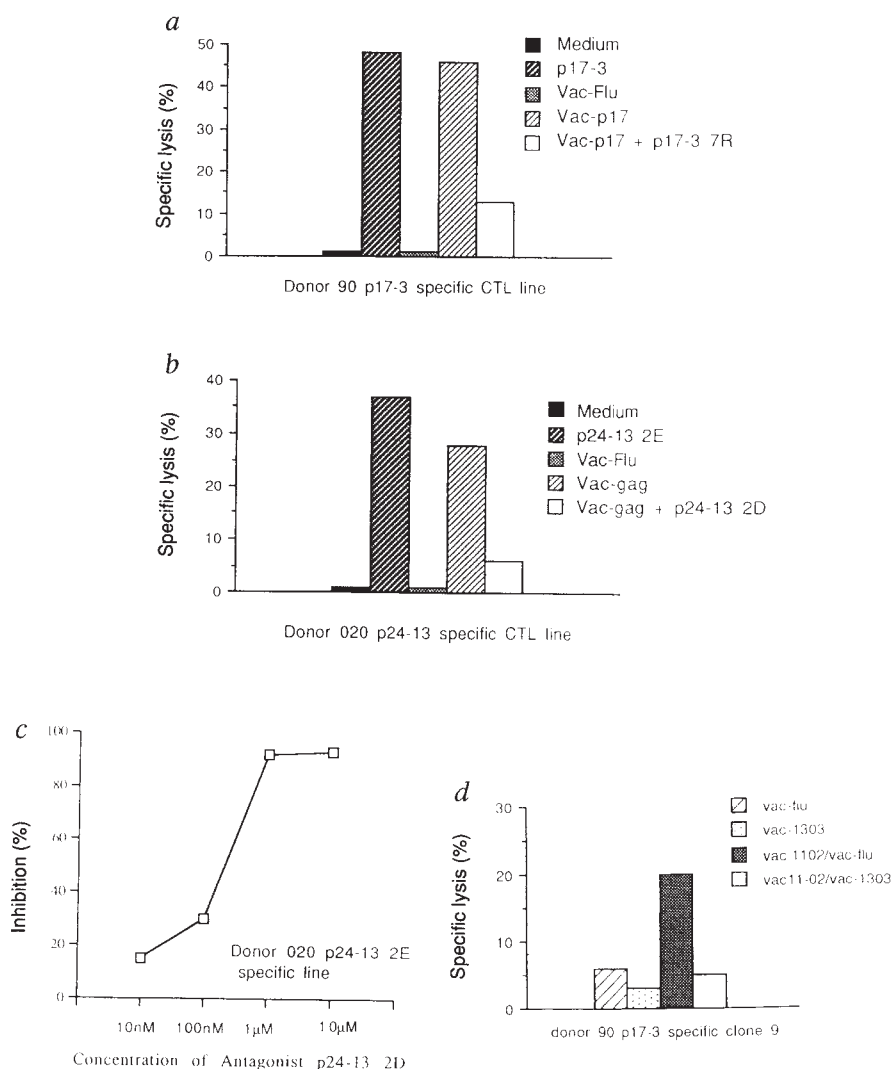
derived from donor 90 were tested in antagonist assays using the p17-3 7R variant (GGKKKYRLK) which was not identified in these donors but is the consensus sequence for African strains and is also the commonest p17-3 variant among North American/European strains<sup>11</sup>. For each HLA-B8-restricted epitope, variant and index peptides bound major histocompatibility complex (MHC) molecules with similar affinities *in vitro* (S.M. *et al.*, manuscript in preparation). Figures 1a, b and c illustrate effective antagonism of CTL by variants at three epitopes: in each case, the variant and the CTL were derived from the same donor.

The common 7R variant of p17-3 also acted as an antagonist for CTL clones derived from donor 90 which failed to lyse p17-3 7R-pulsed targets (Fig. 1d). The three clones shown all had, when sequenced, a single TCR V $\alpha$  and V $\beta$  messenger RNA species (distinct in each case), indicating that antagonism is demonstrable in T cells bearing a single TCR (W.R. *et al.*, manuscript in preparation). Further control experiments con-

firmed previous observations that the effect requires interaction between TCR and peptide bound to MHC on the target cell surface and is not simply due to displacement of bound index peptide by variant peptide or to CTL:CTL interactions<sup>5–10</sup>. Figure 1e shows inhibition of lysis of p17-3 7K-pulsed targets by antagonist p17-3 7R presented on additional, unlabelled (cold) cells despite the fact that 7R-pulsed targets were not lysed by these effector cells. The unrelated B8-restricted peptide derived from influenza matrix (ELRSRYWAI)<sup>12</sup> did not act as an antagonist for either p17-3- or p24-13-specific CTL (data not shown).

The effect of antagonists on naturally processed epitopes was then investigated. Recombinant vaccinia virus (rVV) 11-02 expresses p17 Gag cloned from an asymptomatic donor bearing HLA-B8 and contains the epitope p17-3 7K. Target cells infected with this recombinant virus were recognized by all p17-3-specific CTL lines tested. However, addition of antagonist peptide p17-3 7R to the assay led to marked inhibition of lysis by a line derived from donor 90 (Fig. 2a). A similar result was

FIG. 2 Effective CTL antagonism of and by naturally processed epitopes. **a**, Antagonism of recognition of p17-3 7K expressed by recombinant vaccinia virus (rVV). rVV11-02 was prepared using p17 gag amplified from the DNA of a HIV-1 seropositive individual bearing HLA-B8 using nested PCR (as in Table 1). M13 clones containing p17-3 sequence GGKKKYKLK (7K) were then further digested with *Nco*I and *Bgl*II, ligated into pSC11 and recombined into vaccinia virus using a BUDR selection protocol<sup>15</sup>. DNA sequence was confirmed and expression demonstrated by western blot. Vaccinia infections were done at 3 PFU per cell followed by 15 h incubation and target labelling with <sup>51</sup>Cr. A vaccinia-influenza nucleoprotein (vac-flu) construct was used to infect control targets. Targets were then used as for peptide pulsed targets and the assay was done in the presence or absence of p17-3 variant 7R (5  $\mu$ M). Subline 20 (donor 90) was used at an E:T ratio of 4:1. An uninfected control target with and without p17-3 7K (5 nM) is also shown. **b**, Antagonism of recognition of p24-13 2E expressed by rVV. Lysis of infected cells by a p24-13 specific CTL line (donor 020, E:T ratio 10:1) was estimated with and without antagonist 2D (1  $\mu$ M). An uninfected control target with and without p24-13 2E (20  $\mu$ M) is also shown. The experimental conditions were similar to those above, with the exception that a vaccinia construct expressing full-length HIV-1 gag was used<sup>2</sup>. Labelled target cells were infected at 10 PFU per cell and incubated for 3 h. **c**, Antagonism of recognition of p24-13 2E expressed by replicating HIV in cell-line CEM. A CEM cell line was stably infected with HIV-MN which bears the p24-13 2E sequence (GEIYKRWII). This virus was derived from supernatant from chronically infected H9 cells (provided by the UK AIDS Directed Programme). CEM cells express HLA-B8 and uninfected cells presented peptide p24-13 2E to donor 020-derived specific CTL (specific lysis = 45%, 50  $\mu$ M p24-13 2E prepulsed. Lysis of CEM cells without peptide = 5%. E:T ratio 15:1.) Corresponding specific lysis of cells infected with HIV-MN was 27%. Inhibition was then estimated in the presence of serial dilutions of antagonist p24-23 2D and antagonism calculated as in Fig. 1. **d**, Antagonism of recognition of naturally



processed p17-3 7K expressed by naturally processed p17-3 5R7R. rVV 13-03 was prepared as for rVV 11-02 but contains the variant sequence GGKKRYRLK as the p17-3 epitope (p17-3 5R7R Table 1). Labelled target cells (donor 90 B cell line) were infected for 1 h with either vac-flu 5 PFU, vac 13-03 5 PFU, vac 1102 5PFU + vac-flu 5PFU or vac 11-02 + vac 13-03 5PFU, and incubated for 3 h. Effectors were p17-3 7K-specific clone 9 from donor 90, E:T ratio 4:1.

obtained using p24-13 2E-specific CTL from donor 020: in this case lysis of cells infected with a rVV expressing p55 Gag was inhibited by addition of peptide variant p24-13 2D (Fig. 2b). Next, CEM cells bearing HLA-B8 were infected with HIV-MN (which contains sequence p24-13 2E). These cells were recognized as targets by p24-13-specific CTL from donor 020 and lysis was inhibited by addition of antagonist 2D (Fig. 2c).

Finally, the ability of a naturally processed epitope to act as an antagonist was determined. A variant peptide p17-3 5R7R (Table 1) derived from donor 009, was expressed in rVV 13-03, which encodes full-length p17 Gag containing this epitope. Co-infection of target cells with a 1:1 mixture of rVV 13-03 and rVV 11-02 led to inhibition of lysis by a p17-3 7K-specific clone compared to co-infection of rVV 1102 and rVV expressing influenza nucleoprotein (Fig. 2d). Thus, naturally processed Gag may be antagonized by variant peptides and naturally processed variants may themselves act as an antagonist.

The mechanism of the antagonism seen here and in previous

reports has not been explained<sup>5-7</sup>. If antagonism results from a weak interaction between TCR and peptide, it may be overcome by increasing the concentration of peptide present (as in the case of agonist/antagonist peptides<sup>5</sup>) or by increasing the time CTLs interact with targets<sup>13</sup>. We therefore analysed the kinetics of lysis by several sublines active against either antagonist or index peptides. Prolonged incubation with antagonists p17-3 7R and 7Q ultimately led to lysis by a p17-3 7K-specific line which caused no significant lysis at 4 h compared to index peptide (Fig. 3a). In a line which had dual specificity for p17-3 7K and 7R, lysis of cells pulsed with p17-3 7R occurred after a delay compared to cells pulsed with p17-3 7K (Fig. 3b). Finally, addition of donor-derived antagonist p17-3 7Q led to a similar phenomenon (Fig. 3c). Thus some antagonistic peptides might induce slower activation of CTL.

CTL antagonism, if it occurs *in vivo*, could represent a unique method of viral escape. Genetic variants arise frequently in HIV-1 as a result of error-prone reverse transcription<sup>14</sup>. If these dis-



played antagonism as demonstrated in these individuals, the effect might benefit the entire population of viral quasiespecies, not just viruses bearing that variant. The presence of antagonistic epitopes on neighbouring cells (such as in a lymph node) could also protect cells containing wild-type virus. The antagon-

ist: agonist ratios used here are similar to those used by others<sup>5</sup> and are relevant to those occurring during the course of infection. Furthermore, it has been established that natural antagonists exist in hepatitis B infection which appear to be active at similar ratios (A. Bertolotti *et al.*, personal communication).

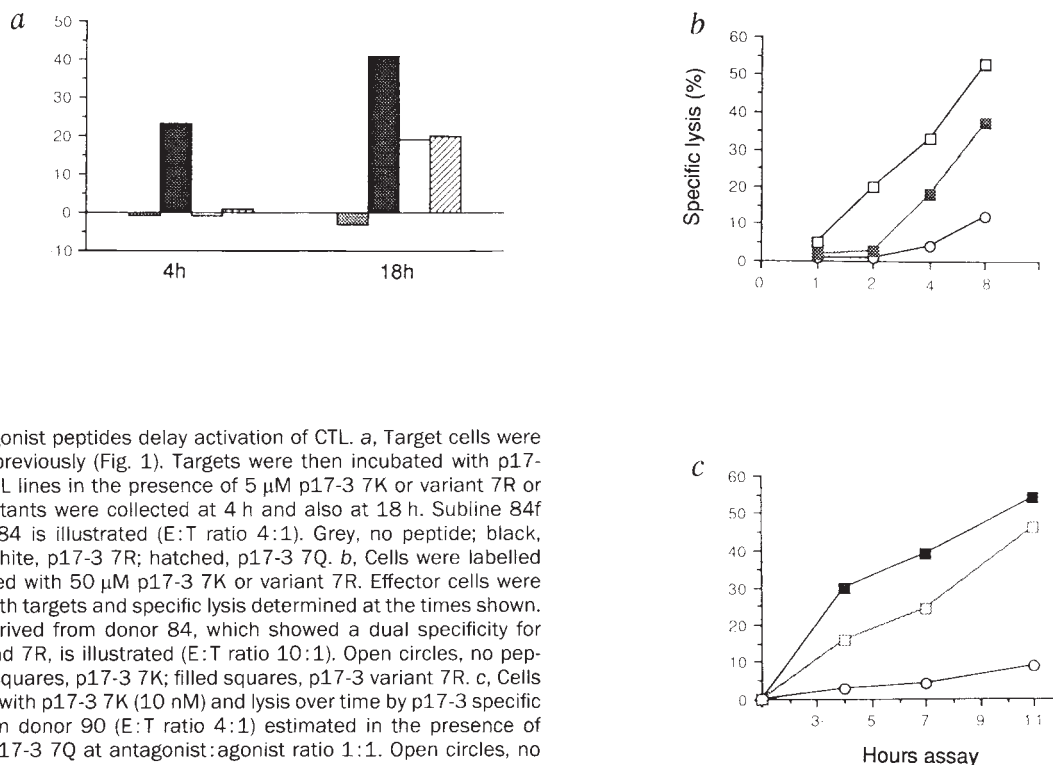


FIG. 3 Antagonist peptides delay activation of CTL. *a*, Target cells were labelled as previously (Fig. 1). Targets were then incubated with p17-3-specific CTL lines in the presence of 5  $\mu$ M p17-3 7K or variant 7R or 7Q. Supernatants were collected at 4 h and also at 18 h. Subline 84f from donor 84 is illustrated (E:T ratio 4:1). Grey, no peptide; black, p17-3 7K; white, p17-3 7R; hatched, p17-3 7Q. *b*, Cells were labelled and prepulsed with 50  $\mu$ M p17-3 7K or variant 7R. Effector cells were incubated with targets and specific lysis determined at the times shown. Line 84a derived from donor 84, which showed a dual specificity for p17-3 7K and 7R, is illustrated (E:T ratio 10:1). Open circles, no peptide; empty squares, p17-3 7K; filled squares, p17-3 variant 7R. *c*, Cells were pulsed with p17-3 7K (10 nM) and lysis over time by p17-3 specific CTL line from donor 90 (E:T ratio 4:1) estimated in the presence of antagonist p17-3 7Q at antagonist:agonist ratio 1:1. Open circles, no peptide; filled squares, p17-3 7K prepulse; empty squares, p17-3 7K prepulse + p17-3 7Q (1:1).

TABLE 1 Naturally occurring variants of HIV-1 Gag epitopes

| HIV source                 | CTL epitope | Agonist sequence (Ag) | Antagonist sequence (Ant) | Proportion<br>Ant/(Ant + Ag)* |       |
|----------------------------|-------------|-----------------------|---------------------------|-------------------------------|-------|
| HLA-B8-restricted epitopes |             |                       |                           |                               |       |
| Donor 90                   | p17-3       | GGKKKYKLK (7K)        | GGKKKYQLK (7Q)            | 6/15                          | 14/29 |
| Donor 84                   | p17-3       | GGKKKYKLK (7K)        | GGKKKYQLK (7Q)            | ND                            | 9/24  |
| Donor 009                  | p17-3       | GGKKKYKLK (7K)        | GGKKRYRLK (5R7R)          | 2/2                           | ND    |
| Donor 020                  | p24-13      | GEIYKRWII (2E)        | GDIYKRWII (2D)            | 22/69                         | ND    |
| Los Alamos                 | p17-3       | GGKKKYKLK (7K)        | GGKKKYRLK (7R)            | 7/15 strains                  |       |
| HLA-B27-restricted epitope |             |                       |                           |                               |       |
| Donor 007                  | p24-14      | KRWIIMGLNK (6M)       | KRWIILGLNK (6L)           | 2/20                          |       |

Sequence of Gag epitopes in donor blood samples. Full-length p17 gag was amplified from the DNA of peripheral blood mononuclear cells or plasma RNA<sup>4,15</sup>. Primers for the PCR were: 5-GTTCTAGGTGATATGG-3, 5-ACTAGCGGAGGCTAG-3 for primary reaction; 5-GCTAG-AATTCATGGGTGCGAGAGCGT-3, 5-CAGTTCTAGATCTAGTAATTTGGCTGACC-3 for the secondary reaction. Genomic DNA (1  $\mu$ g) was added to buffer containing 2.5 units Taq polymerase, 0.05 M KCl, 0.001 M Tris pH 8, 0.0015 M MgCl<sub>2</sub> and 0.2 mM each of dNTPs. Cycling conditions were 5 mins at 94 °C followed by 30 cycles of 1' each at 94, 48 and 72 °C for the primary reaction. 1  $\mu$ l of the primary product was used in the secondary reaction, for which the conditions were identical but the concentration of MgCl<sub>2</sub> was 0.003 M and the annealing temperature was 60 °C. The product was purified, digested with EcoRI and Xba, directionally cloned into M13 and sequenced<sup>4,16</sup>. Plasma RNA was obtained from both donors 90 and 84 according to the protocol of Zhang and colleagues<sup>16</sup>, using the 3' outer primer shown above to initiate cDNA synthesis. Cloning into M13 and sequencing were then as for proviral DNA. Sequence of p24 epitopes was derived from proviral DNA only as previously described<sup>4</sup>. Patient information. Donor 90 has HIV-1 heterosexually acquired in Africa. He is asymptomatic although his CD4 count is  $40 \times 10^6$  l<sup>-1</sup>. Donor 84 is a British male homosexual who seroconverted in Dec 1991 and remains well (CD4 count  $540 \times 10^6$  l<sup>-1</sup>). Donors 020, 009 and 007 are haemophiliacs described previously<sup>4</sup>, the most recent CD4 counts being 90, 20 and  $360 \times 10^6$  l<sup>-1</sup>, respectively.

\* Frequency of antagonist expressed as a proportion of total sequences containing either agonist or antagonist sequence in proviral DNA or plasma RNA. Estimates made for the donors shown, except in the case of 7R.

ND, not done.

In addition the demonstration here of CTL antagonism of and by naturally processed proteins, including Gag expressed by HIV-infected cells, gives this phenomenon further *in vivo* relevance. □

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1. Walker, B. D. *et al.* *Science* **240**, 64–66 (1988).
2. Nixon, D. F. *et al.* *Nature* **336**, 484–486 (1988).
3. Nixon, D. F. & McMichael, A. J. *AIDS* **5**, 1049–1059 (1991).
4. Phillips, R. E. *et al.* *Nature* **354**, 453–459 (1991).
5. Jameson, S. C., Carbone, F. R. & Bevan, M. J. *J. exp. Med.* **177**, 1541–1550 (1993).
6. Evavold, B. D., Sloan-Lancaster, J. & Allen, P. *Immunol. Today* **14**, 602–609 (1993).
7. De Magistris, M. T. *et al.* *Cell* **68**, 625–634 (1992).
8. Ostrov, D. J. *et al.* *J. Immun.* **150**, 4277–4283 (1993).
9. Evavold, B. D. & Allen, P. M. *Science* **252**, 1308–1310 (1991).
10. Racioppi, L. *et al.* *J. exp. Med.* **177**, 1047–1060 (1993).
11. Myers, G. *et al.* (eds) *Human retroviruses and AIDS* (Theoretical Biology and Biophysics group T-10, Los Alamos, 1992).
12. Sutton, J. *et al.* *Eur. J. Immun.* **23**, 447–453 (1993).
13. Hogquist, K. A. *et al.* *Cell* **76**, 17–27 (1994).
14. Dougherty, J. P. & Temin, H. M. *J. Virol.* **62**, 2817–2822 (1988).
15. Maniatis, T., Fritsch, E. F. & Sambrook, J. *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbour Laboratory Press, New York, 1982).
16. Zhang, G. Q. *et al.* *AIDS* **5**, 675–681 (1991).
17. Rowland-Jones, S. *et al.* *Eur. J. Immun.* **23**, 1999–2004 (1993).
18. Mackett, M., Smith, G. L. & Moss, B. J. *J. Virol.* **49**, 857–864 (1984).

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## Natural variants of cytotoxic epitopes are T-cell receptor antagonists for antiviral cytotoxic T cells

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It has been suggested that mutations within immunodominant cytotoxic T-lymphocyte (CTL) epitopes may be exploited by viruses to evade protective immune responses critical for clearance<sup>1–4</sup>. Viral escape could originate from passive mechanisms, such as mutations within crucial CTL epitopes, either affecting major histocompatibility complex binding or T-cell antigen receptor (TCR) recognition. Additionally, it has recently been shown that substitutions of TCR contact sites can yield analogue peptides that can still interact with the T-cell receptor but be unable to deliver a full stimulatory signal, thus inducing anergy<sup>5</sup> or acting as an antagonist for the TCR<sup>6–8</sup>. We report here that hepatitis B virus isolates derived from two chronically infected patients display variant epitopes that act as natural TCR antagonists with the capacity to inhibit the CTL response to the wild-type epitope. During natural infection, TCR antagonist mutations of CTL epitopes could contribute to the development of viral persistence, especially if the antiviral CTL response is monospecific or the epitope is strongly immunodominant.

Residues 18–27 of the hepatitis B virus (HBV) nucleoprotein contain an HLA-A2-restricted CTL epitope<sup>9</sup>, and an efficient response against it and other epitopes derived from the viral

nucleocapsid<sup>10</sup>, envelope<sup>11</sup> and polymerase (F.V.C. *et al.*, manuscript in preparation) proteins is observed in patients who spontaneously clear the virus but not in patients chronically infected by HBV<sup>10–13</sup>. We studied two HLA-A2-positive chronic patients who demonstrated a vigorous CTL response against hepatitis B core (HBc)18–27 despite their inability to clear infection. Both patients were infected with homogeneous populations of viruses with mutations at residues 21 and 27, which could decrease the HLA-A2 binding affinity of the variant peptide (substitution 27) as well as TCR recognition (substitution 21) (Fig. 1, and manuscript in preparation).

These substitutions were not detected in five additional HLA-A2-positive patients with chronic hepatitis B who did not show CTL activity against the epitope HBc18–27 and in ten HLA-A2-negative chronic B patients, all derived from our geographical area.

In the experiments described here, we examined whether these substitutions may have resulted in variant sequences with an antagonistic effect on TCR recognition of the original peptide. More specifically, a panel of HLA-A2-restricted, HBc18–27-specific CTL clones was used to test the inhibitory capacity of variant peptides, carrying amino-acid substitutions at position 21, on lysis of target cells prepulsed with wild-type peptide. This assay has been used previously<sup>6,8</sup> to measure TCR antagonism and discriminate it from competition for major histocompatibility complex (MHC) molecules in both class I- and class II-restricted systems. Different CTL clones showed different susceptibility to the inhibitory activity of the variant peptides. When expressed, inhibition was already detectable at peptide concentrations as low as 10–100 pM, which are comparable to physiological levels of natural peptides within infected cells<sup>14–16</sup>.

For some CTL clones, variant peptides acted as agonist at high peptide concentrations and antagonist at lower concentrations. This behaviour is well illustrated by the CTL clone G7: inhibition of lysis was maximal at picomolar concentrations of variant peptide (Fig. 1a) but progressively declined in parallel with the appearance of the agonist effect of the variant peptides (Fig. 1b).

Variant peptides behaved as pure antagonists for other T cells, as illustrated by the G8 clone (Fig. 1), which was unable to recognize variant peptides even at high doses and was strongly inhibited at a wide range of peptide concentrations (from 10 pM to 100 nM). Clones such as M4 were similarly affected, with the difference of a slight decline in the inhibitory effect at high variant peptide concentrations (100 nM) that were weakly stimulatory in the direct agonist assay (Fig. 1).

Finally, variant peptides were pure agonist for other CTL clones such as G13 (Fig. 1), because they were able to sensitize target cells for CTL lysis and did not inhibit wild-type peptide recognition.

The different sensitivity to the inhibitory effect of the variant peptides shown by distinct CTL clones, some of them being strongly inhibited and others completely insensitive despite a comparable affinity for wild-type peptide/HLA complexes, as demonstrated by their dose-response curves, further demonstrates that these natural variants do not simply exert their inhibitory activity by competing with wild-type peptides for HLA binding. Accordingly, it was found that a peptide unrelated to HBc18–27, but with similar HLA-A2.1 binding affinity, failed to exert any inhibitory effect on HBc18–27-specific CTL clones (Fig. 2a).

Moreover, inhibition of CTL function was observed only when the variant peptide was preincubated with the target cells but not when effector CTL were exposed to it before the chromium release assay. These results rule out the possibility that CTL–CTL lysis (caused by peptide presentation by HLA molecules expressed on the CTL surface) was responsible for the inhibitory effects observed (Fig. 2b). This confirms also that wild-type peptide and variant peptides must be presented by the same target cell for TCR antagonism to occur<sup>17</sup>.

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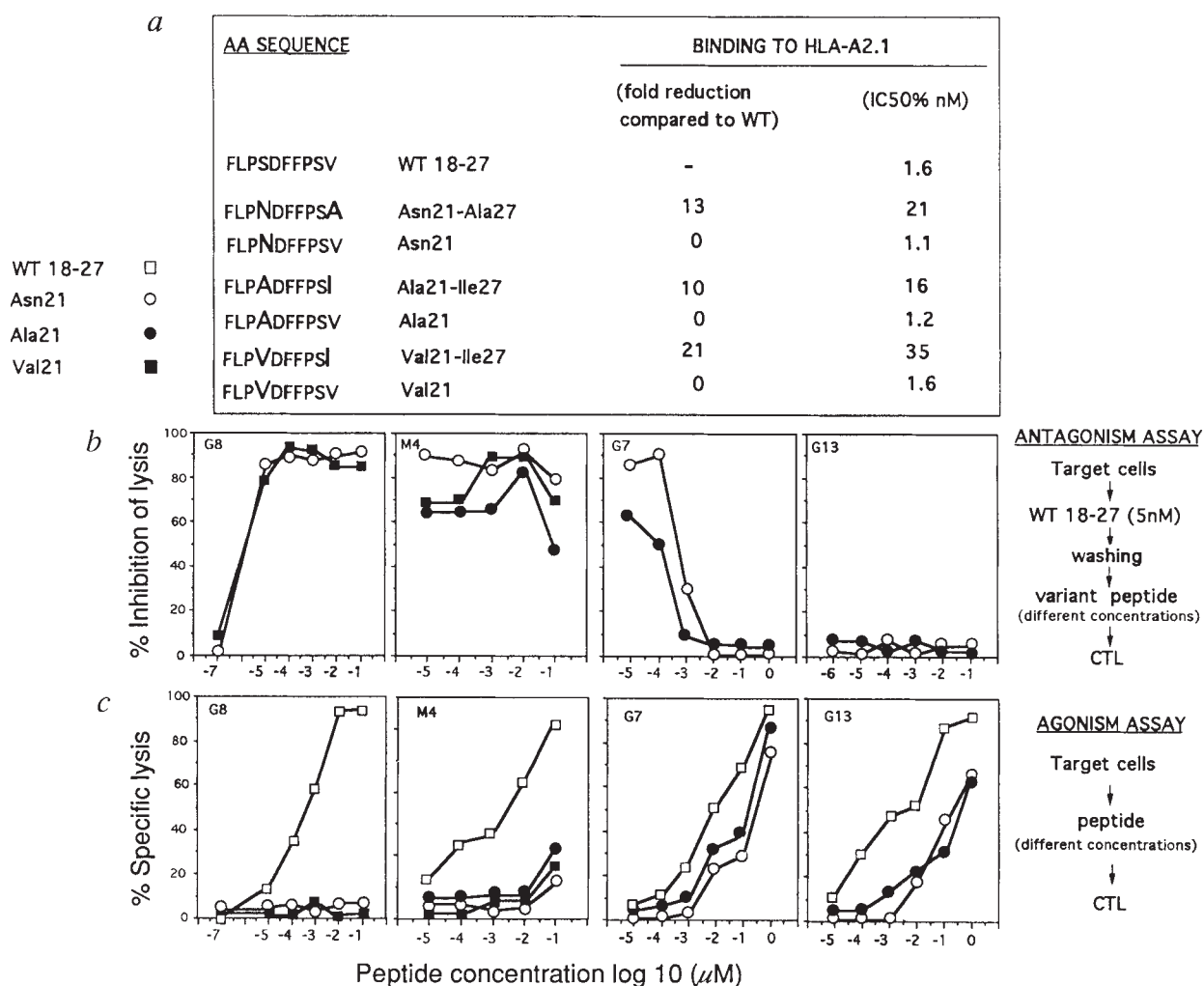
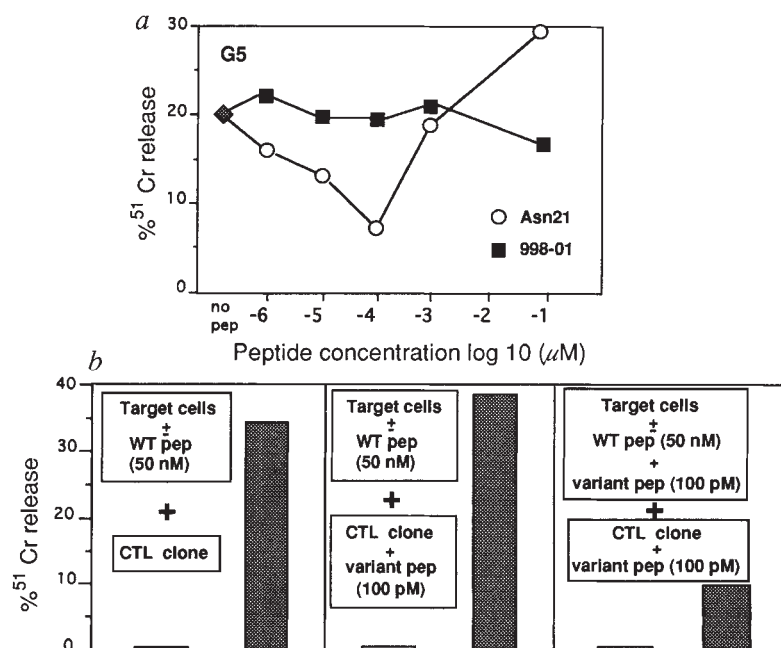


FIG. 1 Influence of natural HBV variants on CTL recognition of the HBcAg sequence 18–27. **a**, Amino-acid sequences (single-letter code) of wild-type (WT) and variant peptides and binding affinity to HLA-A2.1. In a first patient with chronic active hepatitis B, all sequenced HBV genomes showed mutations leading to Asn 21–Ala 27 substitutions (30 DNA clones); a second chronic patient was infected by a viral population with constant Val→Ile substitution at position 27 and Ser to either Ala (75% of the sequenced clones) or Val (25% of the sequenced clones) at position 21. **b**, Clone-specific inhibitory effect of different concentrations of variant peptides on lysis of target cells prepulsed with suboptimal concentrations of wild-type peptide (antagonist effect). **c**, CTL recognition of wild-type and variant peptides (agonist effect).

**METHODS.** CTL clones were produced by limiting dilution (initial cloning at 1 or 10 cells per well followed by subcloning at 0.5 cell per well) from HBV-specific CTL lines derived from 2 patients with acute self-limited HBV infection by initial peripheral blood lymphomononuclear cell (PBMC) stimulation with wild-type HBc18–27 peptide. All clones were able to recognize peptide HBc18–27 as well as endogenously synthesized HBcAg (induced by infection or transfection of target cells with high efficiency recombinant vectors<sup>13</sup>) in the context of HLA-A2 molecules. Synthetic peptides were purchased from Chiron Mimotopes (Clayton, Australia) and were greater than 90% pure after high pressure liquid chromatography. An identical antagonist effect was observed using 98–99%-pure peptides, as analysed by ion-spray mass spectrum analysis. To determine the nucleotide sequence of the HBV precore-core region (nucleotides 1,774 to 2,063, according to ref. 28), polymerase chain reaction (PCR) products (sense primer 1,784–1,802; anti-sense primer 2,063–2,044) were cloned into pUC18 and the recombinant clones were subjected to a standard dideoxy chain termination method using a fluorescent M13 primer with an automated laser DNA sequencer (A.L.F. Sequencer, Pharmacia L.K.B.). The quantitative

assay used for HLA-A2.1 binding peptides based on the inhibition of binding of a radiolabelled standard peptide to detergent-solubilized MHC molecules is described elsewhere<sup>29</sup>. Binding is expressed as IC<sub>50</sub> (50% inhibitory concentration) that represents the dose of competitor peptide yielding 50% inhibition of radiolabelled peptide binding. The study of the antagonist and agonist effects of the variant peptides was done with a standard 4-h <sup>51</sup>Cr release assay using U-bottomed 96-well plates. Briefly, to study antagonism HLA-A2.1 EBV-B cells were labelled with <sup>51</sup>Cr for 1 h and during this time of incubation suboptimal concentrations (5 and 10 nM) of wild-type peptide were added to the culture. After washing, the cells were pulsed with different concentrations of variant peptides or medium alone. After 1 h at 37 °C, CTL were added to target cells at an E/T ratio of 2/1. Results obtained with 5 nM wild-type peptide are illustrated. The specific lysis by CTL clones in the absence of variant peptides was: G8, 90%; M4, 26%; G7, 24%; G13, 35%. In control experiments designed to avoid the direct contact of free peptide with CTL, target cells were washed extensively after incubation with the variant peptide, before addition of the effector CTL. Equivalent levels of inhibition were observed also at these experimental conditions (data not shown). To study recognition of variant and wild-type peptides by CTL (agonist assay), target cells were incubated with graded concentrations of the individual peptides during the time of <sup>51</sup>Cr labelling, then washed and co-cultured with effector CTL. Radioactivity in the culture supernatants was counted on a Matrix 96 (Canberra Packard, Meriden, CT, USA). Per cent specific lysis was calculated with the following formula:  $100 \times ((\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release}))$ . Spontaneous release was always <20% in all assays. Percentage inhibition of lysis was calculated as follows:  $100 \times ((A - B) / A)$ , where A is the per cent specific lysis in the absence of mutated peptides and B is the per cent lysis in its presence. Each point represents the mean of duplicate wells.

FIG. 2 a, Effect of variant peptides and an HBV unrelated peptide on CTL lysis of HLA-A2-positive target cells prepulsed with HBc18-27 (5 nM) (culture conditions as described in the legend to Fig. 1). The HBV-unrelated peptide 998-01 (FLYCYFALV) and the HBc18-27 peptide have HLA-A2.1 binding affinity within the same range. b, Effect of CTL preincubation with the variant peptides. HLA-A2-positive target cells were pulsed with or without suboptimal concentrations of wild-type peptide (50 nM) during the time of  $^{51}\text{Cr}$  labelling (1 h) and then washed. Left, wild-type peptide prepulsed target cells were co-cultured with effector CTL (clone G7). Middle, wild-type peptide prepulsed target cells were cocultured with effector CTL previously incubated for 1 h with variant (Asn 21) peptide and then washed before addition to target cells. Right, wild-type peptide prepulsed target cells were incubated for an additional hour with variant (Asn 21) peptide (100 pM) and then washed before co-culture with effector CTL previously pulsed for 1 h with variant (Asn 21) peptide (100 pM). The E/T ratio was 2/1 in all experimental conditions. Targets with (shaded) or without (unshaded) wild-type peptide.



To mimic more closely the intracellular events occurring during viral infections *in vivo*, we tested the inhibitory effect of variant analogues on CTL recognition of natural peptides generated by intracellular processing of endogenously synthesized HBcAg. For this purpose, variant peptides were incubated with HLA-A2-positive cells transfected with a plasmid carrying the entire HBV core gene (ayw subtype) which represent efficient targets for 18-27-specific CTL clones<sup>18</sup>. CTL recognition of core peptides derived from endogenous processing was inhibited by peptides with a single substitution at 21 as well as peptides with a double 21-27 substitution (Fig. 3).

Antagonist effect by the double 21-27 substitution was usually weaker (lower level of inhibition at equimolar peptide concentrations and narrower range of inhibitory concentrations) and less reproducible (4 experiments, out of a total of 11 assays in which the double and single substitutions were used in parallel, with no antagonist effect exerted by the double 21-27 substitution, despite effective inhibition induced by the 21 variant) than that expressed by analogue peptides with substitution 21 alone. The roughly 10- to 20-fold reduction of HLA binding affinity caused by the conservative substitution of valine 27 with alanine or isoleucine (found in HBV mutants) probably accounts for this weaker antagonist effect. By contrast, the fact that these variant peptides still remain good HLA-A2 binders, despite substitution of a major anchor residue, would explain why the antagonist activity is not completely lost.

Finally, the effect of the variant peptides on other effector functions of the CTL clones was investigated by measuring the amount of interferon- $\gamma$  (IFN- $\gamma$ ) secreted by CTL stimulated by wild-type 18-27 peptide in the presence or absence of the mutated peptide. IFN- $\gamma$  was studied because it is the only detectable lymphokine secreted by HBc18-27-specific CTL clones on peptide recognition and because IFN- $\gamma$  can amplify the cytopathic effect of the CTL by recruiting antigen nonspecific inflammatory cells at the site of antigen synthesis<sup>19</sup>. Inhibition of IFN- $\gamma$  secretion in the presence of mutated peptides paralleled the effect on target-cell lysis, showing that the antagonist effect is not limited to cytotoxicity but can also affect other CTL effector functions (Fig. 4). Because supernatants were tested 24 h after peptide stimulation, the inhibitory effect seems to be long-lasting. This long duration is not caused by anergy induction, because CTL recovered after pre-culture with target cells expressing both wild-type and mutated peptides display levels of CTL activity against wild-type peptide-pulsed target cells identical to those shown by

CTL pre-cultured with target cells expressing wild-type peptide alone (data not shown).

If localized TCR crosslinking is needed to initiate intracellular signalling, as recently proposed for HLA class II-restricted responses, a low-affinity interaction of specific TCRs with variant peptide-HLA complexes could preclude stable and efficient TCR multimerization, thereby inhibiting proper signal transduction<sup>20-22</sup>. In view of this interpretation, it is quite surprising that picomolar concentrations of variant peptide can efficiently inhibit wild-type peptide concentrations 100 times

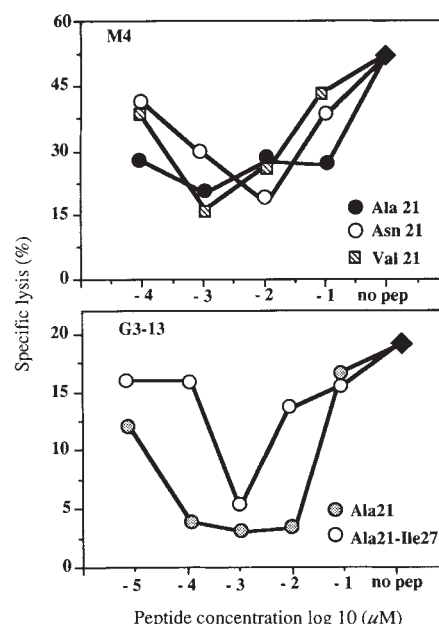
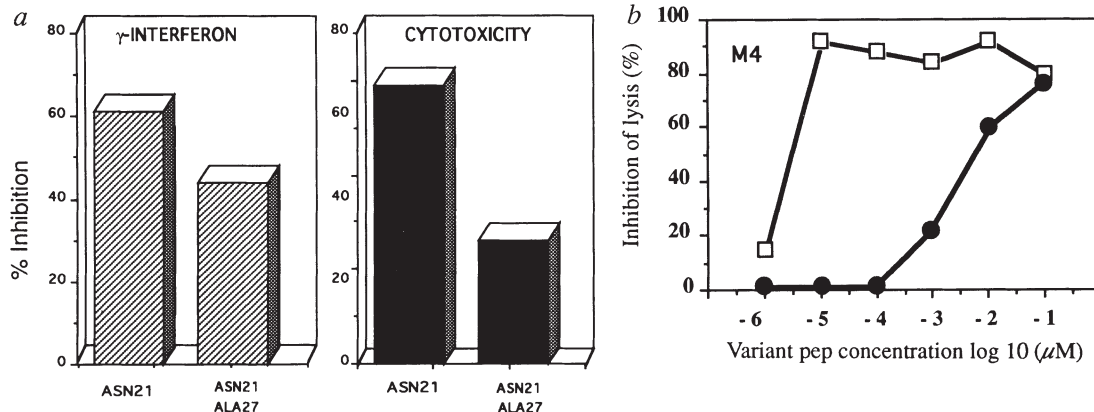


FIG. 3 Inhibitory effect of natural variant peptides on CTL recognition of endogenously synthesized HBV core antigen. Target cells were HLA-A2.1-positive EBV-B cells transfected with the EBV-based plasmid vector EBO-pLPP carrying the HBV core gene (EBO-core). Control experiments were with target cells transfected with an EBO vector carrying the polymerase gene. The construction and characterization of these vectors have been reported in detail elsewhere<sup>18</sup>.  $^{51}\text{Cr}$ -labelled EBO-core targets were incubated with the indicated concentrations of variant peptides. CTL clones were added at an E/T ratio of 3/1. CTL lysis of control target cells transfected with the polymerase gene was 4%.



FIG. 4 a, Parallel inhibition of IFN- $\gamma$  production and cytotoxic activity by natural variant peptides. EBV-B cell targets were incubated with 10 nM wild-type (WT) peptide for 1 h at 37 °C. After two washing steps, EBV-B cells were incubated with 1 nM or 100 pM of the indicated variant peptides for an additional 1 h. After two further washings,  $5 \times 10^3$  target cells were added to the wells containing  $3 \times 10^4$  CTL from clone G5. After 24 h incubation, the plate was centrifuged and 100  $\mu$ l of supernatants were removed. IFN- $\gamma$  production was quantified by a commercial radioimmunoassay (Centocor Inc., Malvern, PA). Experiments with 100 pM antagonist peptides are shown. Similar results were obtained using higher concentrations of variant peptides (1 nM). The level of IFN- $\gamma$  production without antagonist peptides was  $46 \text{ U ml}^{-1}$ . b, Influence of culture conditions on the antagonist effect of the variant peptide.  $^{51}\text{Cr}$ -labelled EBV-B cell targets were incubated with a suboptimal concentration of wild-type peptide (10 nM) for 1 h, then washed and pulsed



with different concentrations of variant peptide (Asn 21) or medium alone; after 1 h incubation, CTL were added to target cells (experimental condition 1;  $\square$ ). In parallel,  $^{51}\text{Cr}$ -labelled EBV-B cell targets were simultaneously incubated with a suboptimal concentration of wild-type peptide (10 nM) and different concentrations of variant peptide (Asn 21); after 1 h incubation, CTL were added to target cells (experimental condition 2;  $\bullet$ ). Lysis of target cells incubated with wild-type peptide alone was 26% and 20% for experimental conditions 1 and 2, respectively. The E/T ratio was always 2/1.

higher. The dissociation of at least certain determinants from HLA class I molecules on intact, viable cells at 37 °C is relatively rapid<sup>16</sup>, and furthermore, when the kinetics of dissociation of preformed complexes between HbC18–27 and purified A2.1 were examined *in vitro*, a  $t_{1/2} \leq 1$  h at 37 °C was measured for this particular peptide–MHC combination<sup>23</sup>. In this context, it is possible that the actual ratio of wild-type/antagonist antigen present in the assay may be lower than expected, as a result of dissociation of antigen/HLA-A2 complexes during the antagonist pulse. If this were to be the case, one would predict that the inhibitory concentrations of variant peptide must be higher if wild-type and variant peptides are added together to target cells. Indeed, this seems to be the case because, under these experimental conditions, inhibition becomes optimal when an antagonist excess or an equimolar concentration of both peptides is present in culture (Fig. 4b). In any case, we should emphasize that the extreme potency of the antagonist effect detected with the single substitution-21 analogue (and, to a lesser degree, the naturally occurring analogue) is somewhat surprising, in the quantitative sense, in light of previous work<sup>8</sup>. Future studies will have to analyse more thoroughly the molecular mechanisms involved in this effect. The clear fact emerging from these and other studies (P. Klennerman and A. J. McMichael, personal communication), however, is that natural variants can act as powerful and specific inhibitors of naturally occurring antiviral cytotoxic T cells.

We have identified natural amino-acid substitutions within the core molecule of HBV strains infecting patients with chronic HBV infection that can affect CTL function through the antagonistic effect of variant peptide–HLA complexes on TCR recognition of the wild-type peptide. Among a population of T-cell clones with a wide spectrum of reactivity against the variant peptides (ranging from null to efficient recognition), more than 80% (11 out of 13) are susceptible to antagonism, suggesting that these observations may be pathogenetically relevant. The antagonist mutants will decrease the probability that the cell in which the mutation arose will be eliminated by the CTL population(s) susceptible to antagonism. This phenomenon may be of pathological consequence if the antiviral CTL response is focused on the critical epitope, either because the response is monospecific or because the epitope is strongly immunodominant. Antagonism could thus represent a critical force to suppress wild-type epitope recognition following the generation of variant HBV DNA. Because an individual cell generally contains tens

or hundreds of HBV DNA copies<sup>24</sup>, which arise through amplification of DNA synthesis by recycling of HBV cores to the nucleus<sup>25</sup>, a situation can be envisaged in which individual infected cells express simultaneously variant and wild-type peptides on their surface. This will fulfill the molecular requirements for antagonism to occur, effectively protecting the cell containing the mutant virus from immune elimination by CTL specific for the wild-type HbC18–27 epitope. This mechanism might also lead to selective expansion of the variant viral population due to the survival of cells that are infected by the variant, either exclusively or together with the wild-type virus, as long as they are not efficiently destroyed by CTL with other specificities. Under such conditions this mechanism could represent a powerful factor for the development of persistent infection by the hepatitis B virus, and perhaps by other viruses that display uncommonly high mutations rates, such as the human immunodeficiency virus<sup>26</sup> and the hepatitis C virus<sup>27</sup>.  $\square$

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- Pircher, H. et al. *Nature* **346**, 629–633 (1990).
- Phillips, R. E. et al. *Nature* **354**, 453–459 (1991).
- de Campos-Lima, P. O. et al. *Science* **260**, 98–100 (1993).
- McMichael, A. J. *Science* **260**, 1771–1772 (1993).
- Sloan-Lancaster, J., Evavold, B. D. & Allen, P. M. *Nature* **363**, 156–159 (1993).
- De Magistris, M. T. et al. *Cell* **68**, 625–634 (1992).
- Racioppi, L., Ronchese, F., Matis, L. A. & Germain, R. N. *J. exp. Med.* **177**, 1047–1060 (1993).
- Jameson, S. C., Carbone, F. R. & Bevan, M. J. *J. exp. Med.* **177**, 1541–1550 (1993).
- Bertoletti, A. et al. *J. Virol.* **67**, 2376–2380 (1993).
- Missale, G. et al. *J. exp. Med.* **177**, 751–762 (1993).
- Nayersina, R. et al. *J. Immun.* **150**, 4659–4671 (1993).
- Penna, A. et al. *J. exp. Med.* **174**, 1565–1569 (1990).
- Bertoletti, A. et al. *Proc. natn. Acad. Sci. U.S.A.* **88**, 10445–10449 (1990).
- Hunt, D. F. et al. *Science* **255**, 1261–1263 (1992).
- Falk, K. et al. *J. exp. Med.* **174**, 425–434 (1991).
- Christinck, E. R., Luscher, M. A., Barber, B. H. & Williams, D. B. *Nature* **352**, 67–70 (1991).
- Ruppert, J. et al. *Proc. natn. Acad. Sci. U.S.A.* **90**, 2671–2675 (1993).
- Guilhot, S. et al. *J. Virol.* **66**, 2670–2678 (1992).
- Ando, K. et al. *J. exp. Med.* **178**, 1541–1554 (1993).
- Brown, J. H. et al. *Nature* **364**, 33–39 (1993).
- Pleogh, H. & Benaroch, P. *Nature* **364**, 16–17 (1993).
- Symer, D. E., Dintzis, R. Z., Diamond, D. J. & Dintzis, H. M. *J. exp. Med.* **176**, 1421–1430 (1992).
- Sette, A. et al. *Molec. Immun.* (in the press).
- Korba, B. E. et al. *J. Virol.* **61**, 1318–1324 (1987).
- Tuttleman, J., Pourcel, C. & Summers, J. *Cell* **47**, 451–460 (1986).
- Hu, W.-S. & Temin, H. M. *Science* **250**, 1227–1233 (1990).
- Houghton, M. et al. *Hepatology* **14**, 381–388 (1991).
- Galibert, F. et al. *Nature* **281**, 646–648 (1979).
- Ruppert, J. et al. *Cell* **74**, 1–20 (1993).

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# Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane

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A CONSERVED tyrosine kinase-activated signal transduction pathway has recently been identified that comprises the plasma membrane-bound small guanine-nucleotide-binding protein Ras and the protein kinases Raf, MAP-kinase kinase and MAP kinase<sup>1,2</sup>. GTP-bound Ras interacts directly with the amino-terminal regulatory domain of Raf<sup>3-8</sup>, but although Ras and Raf can be coimmunoprecipitated from ligand-stimulated cells<sup>9,10</sup>, Ras-GTP does not stimulate the kinase activity of Raf *in vitro*<sup>6</sup>. Furthermore, we have failed to detect Ras in preparations of active detergent-solubilized Raf, demonstrating that once it is activated, Raf does not require Ras. Whereas Raf is normally cytosolic, in cells expressing active Ras, Raf is associated with the plasma membrane. This led us to investigate whether Ras is required to localize Raf to the plasma membrane in order for Raf to become activated. We fused the membrane localization signal of K-Ras(4B) to the carboxy terminus of Raf. This protein is constitutively active and can be further activated by epidermal growth factor, independently of Ras. Our results indicate that Ras functions as a regulated, membrane-bound anchor for Raf, and that other signal(s) also contribute to Raf activation.

To investigate whether a continuous interaction with Ras is required for Raf activity, we determined whether active Raf immunoprecipitates contain Ras. COS-1 cells transfected with expression vectors encoding Ras and Raf, both tagged with a c-Myc epitope<sup>11</sup>, were lysed and immunoprecipitated with an anti-Raf serum<sup>12</sup>. After extensive washing, immunoprecipitates were assayed for Raf activity (Fig. 1a) and analysed by western blotting with monoclonal antibody 9E10 to reveal epitope-tagged Raf and Ras (Fig. 1b). No Ras was detected in the active Raf immunoprecipitates (Fig. 1b), although the lysates contained at least five times more Ras than Raf (Fig. 1b; right lane of each panel) and western blotting could detect Ras if the stoichiometry of Ras to Raf was as low as 1:1,000 (data not shown). Thus, once activated, an interaction with Ras is no longer required for Raf activity. Consistent with this, Ras is undetectable in gel-filtration column fractions containing active Raf<sup>13</sup>.

Raf is normally cytoplasmic, but the observation that Raf co-localizes with oncogenic Ras at the plasma membrane of an E1A/*ras*-transformed cell line<sup>14</sup> suggests that Ras may function to localize Raf to the plasma membrane. To investigate this, we studied the localization of Raf in MDCK cells co-expressing epitope-tagged cellular Raf and mutant Ha-Ras (Fig. 2a-d). In control cells, Raf was predominantly cytoplasmic (Fig. 2a, left panel), whereas in cells expressing oncogenic V12Ras, Raf co-localized with Ras at the plasma membrane (Fig. 2b). Significantly, expression of non-membrane-localized V12Ras (V12S186Ras; Fig. 2c), or V12Ras with a mutation in the effector loop so that it does not interact with Raf<sup>5</sup> (V12A38Ras; Fig. 2d), did not result in membrane localization of Raf.

To investigate whether the targeting of Raf to the plasma membrane may lead to its activation, we exploited the fact that C-terminal sequences of Ras can be used to direct heterologous cytoplasmic proteins to the plasma membrane<sup>15</sup>. A vector was

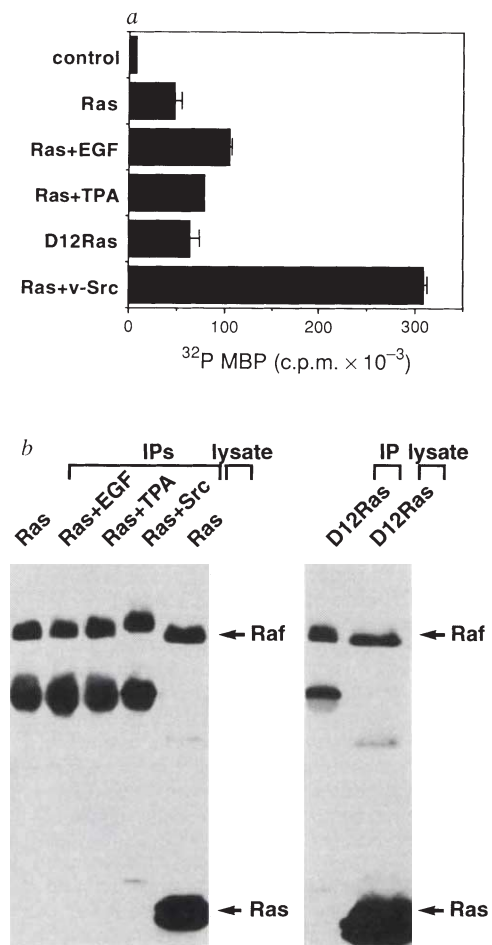


FIG. 1 Absence of Ras in immunoprecipitates of active Raf. Lysates were prepared from COS-1 cells transfected with vectors encoding 9E10 epitope-tagged c-Raf (control), 9E10 epitope-tagged c-Raf and c-Ha-Ras (Ras, Ras + EGF, Ras + TPA), 9E10 epitope-tagged c-Raf and oncogenic Asp12Ha-Ras (D12Ras) or 9E10 epitope-tagged c-Raf and c-Ha-Ras, and v-Src (Ras + Src). Treatment with 10 ng ml<sup>-1</sup> EGF or 40 nM TPA was for 10 min before lysis. a, Raf kinase assays. Equal amounts of Raf (judged by western blotting with 9E10) were immunoprecipitated with a Raf antibody and assayed for activation of MAP-kinase kinase, which in turn was assayed for its ability to activate MAP kinase, measured by phosphorylation of myelin basic protein (MBP)<sup>24</sup>. Results shown are the mean (± average error from mean) of duplicate assays. b, Western blot analysis of Raf immunoprecipitates assayed in a. The amount of c-Ras (left panel) or D12Ras (right panel) relative to active Raf in the immunoprecipitates (IP) was examined by western blotting the anti-Raf immunoprecipitates with 9E10. As a control, half the amount of lysate included in the immunoprecipitation was also analysed (right lane of each panel).

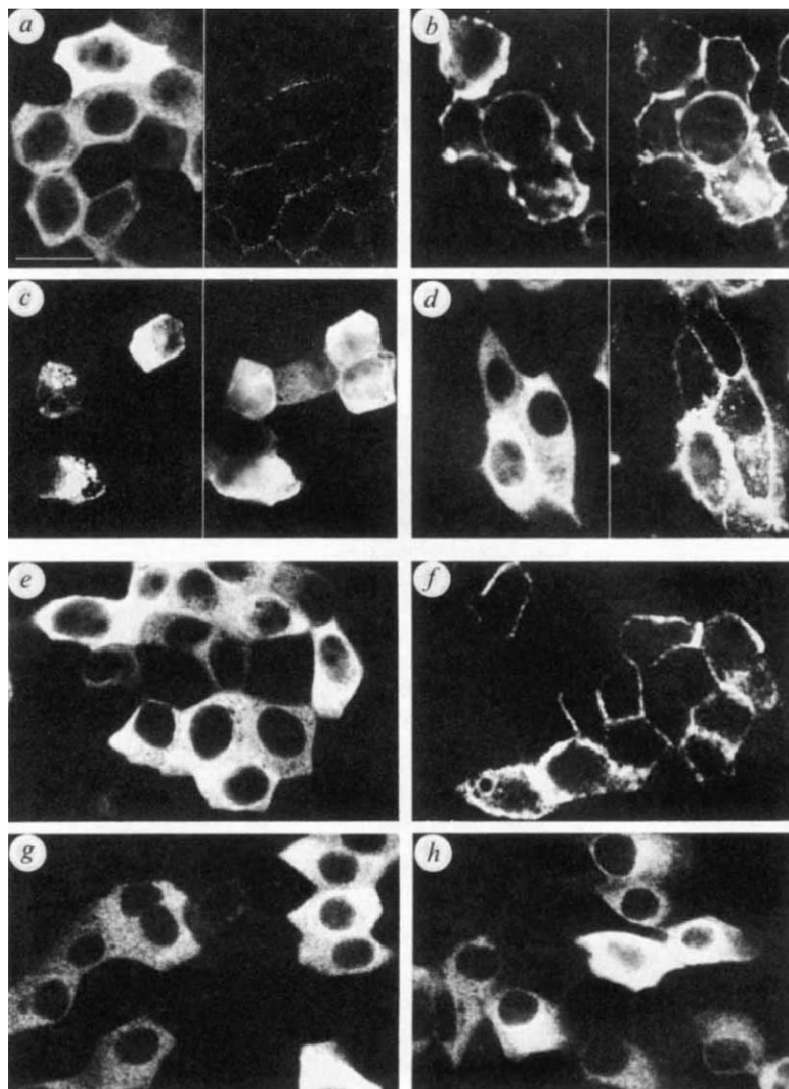
**METHODS.** COS-1 cells were transfected and lysates prepared in S buffer<sup>25</sup> minus SDS, plus 100 nM okadaic acid and 20 mM *N*-octyl-β-D-glucopyranoside (Sigma) as described<sup>25</sup>. Raf was immunoprecipitated by incubating the lysates for 90 min with anti-Raf peptide SP63 antibody<sup>12</sup> pre-bound to protein G-Sepharose beads. The beads were washed three times in lysis buffer minus PMSF, three times in 0.5 M LiCl, 50 mM Tris-HCl, pH 7.5, 5 mM *N*-octyl-β-D-glucopyranoside, and once in 50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.5 mM Na<sub>3</sub>VO<sub>4</sub>, 0.1% 2-mercaptoethanol (buffer A), then incubated for 30 min at 30 °C in 30 μl 30 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.1% 2-mercaptoethanol, 0.03% Brij-35, 10 mM magnesium acetate, 200 μM ATP, 20 mM *N*-octyl-β-D-glucopyranoside, 100 ng μl<sup>-1</sup> of a glutathione S-transferase fusion protein GST-ERK2 (ref. 26), 6.5 ng μl<sup>-1</sup> GST-MAP-kinase kinase<sup>27</sup>. The reaction was stopped by diluting 10 μl into 40 μl ice-cold buffer A plus 1 mg ml<sup>-1</sup> bovine serum albumin, and 10 μl of this was assayed for MBP kinase activity as described<sup>24</sup>. Control, assays from which GST-ERK2 or GST-MAP-kinase kinase was omitted ensured that neither ERK2 nor MAP-kinase kinase coimmunoprecipitated with Raf (data not shown).

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FIG. 2 Intracellular localization of Raf. *a-d*, MDCK cells were stained with monoclonal antibody 9E10 (ref. 11) to reveal epitope-tagged c-Raf (left panels) and with a mixture of monoclonal antibodies Y13-238 and Y13-259 (ref. 28) (right panels) to reveal: *a*, endogenous Ras; *b*, active Ha-Ras (V12Ras); *c*, V12Ras that is not post-translationally modified (V12S186Ras); and *d*, V12Ras with a mutation in the effector loop (V12A38Ras). *e-h*, Localization of Raf to the plasma membrane by Ras membrane-localization signals. MDCK cells were stained using monoclonal antibody 9E10 to reveal: *e*, Raf; *f*, Raf-CAAX; *g*, Raf-SAAX; and *h*, RafK6Q-CAAX. Scale bar, 20  $\mu$ m.

**METHODS.** Confluent MDCK cells growing on plastic Petri dishes in DMEM with 10% fetal bovine serum (FBS) were microinjected with plasmid DNAs (50  $\mu$ g ml<sup>-1</sup>) either singly (*a*, *e*, *f*, *g*, *h*) or in combination (*b*, *c*, *d*), incubated overnight, fixed in 4% formaldehyde, stained for immunofluorescence as described<sup>14</sup> and examined with a BioRad MRC600 confocal imaging system in conjunction with a Nikon Diaphot epifluorescence microscope. To epitope-tag Raf at the N terminus, amplification by polymerase chain reaction (PCR) was performed from pEXV-raf<sup>25</sup> with a forward primer containing a *Bam*HI site, Kozak consensus sequence and sequence encoding the 9E10 epitope tag (MEQLISEEDLK) and the first five amino acids of c-Raf; and a reverse primer encompassing the unique *Pvu*II site at base pair 74 of c-raf. The *Bam*HI/*Pvu*II-digested PCR product was ligated into *Bam*HI/*Eco*RI-digested pBluescript with a *Pvu*II/*Eco*RI fragment containing the rest of the c-raf coding sequence generated from pEXV-raf<sup>25</sup>. To attach the nucleotide sequence encoding the 20 C-terminal amino acids of K-Ras (KMSKDGKKKKKSKTKCVIM) to the 3' end of tagged Raf, PCR amplification was performed from vectors encoding K-Ras, S186K-Ras and K6QK-Ras<sup>16</sup>, with a forward primer encompassing the unique *Ava*I site at base pair 1,925 of c-raf and encoding the eight C-terminal amino acids of Raf and the first five amino acids of the K-Ras C terminus, and a reverse primer comprising the last seven codons of K-ras, a stop codon and an *Eco*RI site. The *Ava*I/*Eco*RI-digested PCR product was ligated with a *Not*I/*Ava*I fragment prepared from the tag-Raf pBluescript vector into the eukaryotic expression vector pmt-SM<sup>29</sup> to generate pmt-SM-Raf-CAAX, pmt-SM-Raf-SAAX and pmt-SM-RafK6Q-CAAX. To generate pmt-SM-Raf, the same ligation was done but with an *Ava*I/*Eco*RI fragment excised from pUC13-containing c-raf (American Type Culture Collection) replacing the PCR product.



constructed to express a Raf protein (Raf-CAAX) with a C terminus comprising the C-terminal 20 amino acids of K-Ras(4B), including the CAAX box required for post-translational modification (farnesylation, proteolysis and carboxymethylation) and the polybasic domain required for the plasma membrane localization of K-Ras<sup>16</sup>. Immunofluorescence of microinjected MDCK cells demonstrated that Raf-CAAX localizes to the plasma membrane (Fig. 2*f*), whereas a control protein, Raf-SAAX, in which the cysteine residue of the CAAX box is changed to serine to prevent farnesylation, was cytoplasmic like Raf itself (Fig. 2*e*, *g*).

Kinase assays of immunoprecipitates revealed that Raf-CAAX is 30-fold more active than Raf in the absence of growth factor stimulation, whereas Raf-SAAX is no more active than Raf (Fig. 3*a*). The constitutive activation of Raf-CAAX was confirmed by demonstrating that Raf-CAAX but not Raf or Raf-SAAX activates the MAP kinase ERK2 in cosK-1 cell transfections and induces neurite outgrowth from PC12 cells (Fig. 3*b*, *c*) and morphological transformation of NIH3T3 cells (data not shown). These data are consistent with ligand-stimulated Ras-GTP formation causing a redistribution of Raf to the plasma membrane in order for Raf to be activated. They also explain the anomaly that post-translationally modified Ras is required to activate the MAP kinase pathway<sup>17</sup>, whereas unmodified Ras can interact with Raf<sup>3-7</sup>.

It has been suggested that a lipid activator may bind to the N-terminal regulatory domain of Raf<sup>18</sup>, raising the possibility that the farnesyl group on Raf-CAAX directly activates the Raf kinase domain. Although it has been shown that a farnesyl analogue does not increase the kinase activity of Raf *in vitro*<sup>13</sup>, we investigated this possibility by constructing a vector to encode RafK6-QCAAX. In this protein the CAAX motif is intact to permit farnesylation and other post-translational modifications, but the six lysine residues of K-Ras required for plasma membrane localization are substituted by non-charged glutamine residues<sup>16</sup>. The majority of RafK6-QCAAX was in a farnesylated cytoplasmic form, with only a small proportion localized to the plasma membrane (Fig. 2*h*). RafK6-QCAAX had an activity 10-fold lower than Raf-CAAX (Fig. 3*a*), showing that Raf-CAAX is activated as a result of plasma membrane localization and not simply farnesylation.

As Raf-CAAX is localized at the plasma membrane close to Ras, its activation might result from enhanced interaction with Ras. We therefore examined the effect of dominant-negative N17Ras<sup>19</sup> expression on Raf-CAAX activity. N17Ras inhibited epidermal growth factor (EGF)-stimulated Raf activity but not Raf-CAAX activity (Fig. 4*a*), or Raf-CAAX-induced PC12 cell neurite outgrowth (Fig. 4*b*), indicating that membrane-localized Raf is activated by a Ras-independent mechanism. It has been shown<sup>20</sup> that maximal activation of Raf in an insect cell expres-

FIG. 3 Membrane-targeted Raf-CAAX is constitutively active. *a*, Kinase activity of Raf proteins expressed in COS-1 cells, immunoprecipitated with 9E10 and assayed as described in the legend to Fig. 1. *b*, Effect of Raf protein expression on ERK2 activity in COS-1 cells measured as incorporation of  $^{32}\text{P}$  into MBP by 'in-gel' renaturation kinase assays. For *a* and *b*, results shown are the mean (+average error from mean) of duplication determinations after subtraction of the assay background, determined by assaying lysates of COS-1 cells transfected with empty vector(s). *c*, Expression of Raf-CAAX (left panel) but not Raf-SAAX (right panel) induces PC12 cell neurite extension. Cells were stained with 9E10. Scale bar, 25  $\mu\text{m}$ .

METHODS. *a*, Lysates prepared from COS-1 cells transfected with pmt-SM-c-Raf, pmt-SM-Raf-CAAX, pmt-SM-Raf-SAAX or pmt-SM-RafK6Q-CAAX and containing equal amounts of the different Raf proteins (as judged by western blotting with 9E10) were assayed for 9E10-immunoprecipitated Raf activity as described in the legend to Fig. 1, except that the LiCl washes of the immunoprecipitates were omitted. *b*, Lysates were prepared from COS-1 cells transfected as in *a*, but including the pEXV-ERK2-tag reporter plasmid<sup>25</sup>, then lysates containing equal amounts of ERK2-tag (judged by western blotting with 9E10) were assayed for renaturable ERK2-tag MBP kinase activity<sup>17</sup>, quantified using a Molecular Dynamics PhosphorImager with ImageQuant software. *c*, Subconfluent PC12 rat pheochromocytoma cells cultured on 'Biocoat' laminin-coated Petri dishes (Becton-Dickinson) in DMEM, 10% horse serum, 5% FBS were microinjected with pmt-SM-Raf-CAAX or pmt-SM-Raf-SAAX DNA (50  $\mu\text{g ml}^{-1}$ ). After 20 h, cells were fixed and immunostained with 9E10 for exogenous Raf expression (as described in the legend to Fig. 1), then Raf-expressing cells were examined for neurite outgrowth.

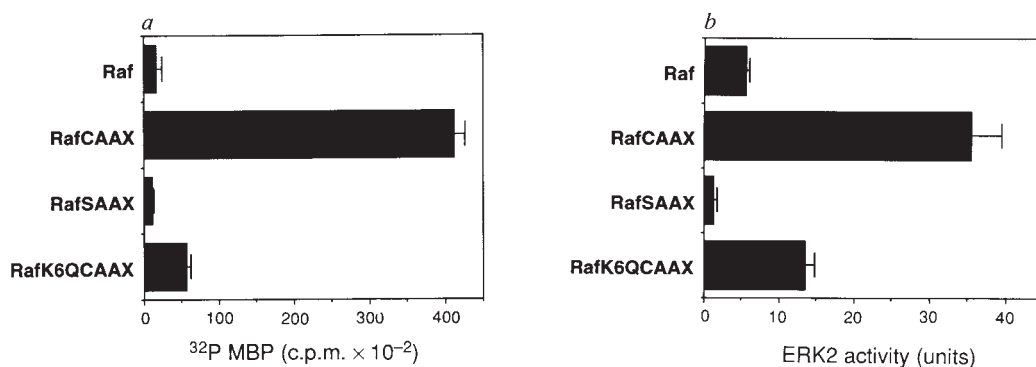
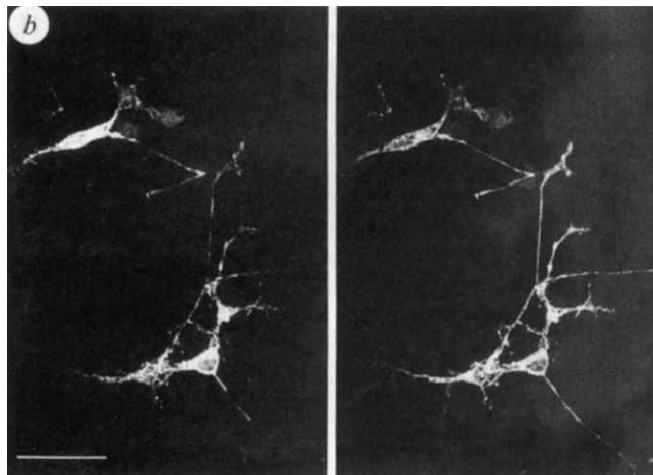
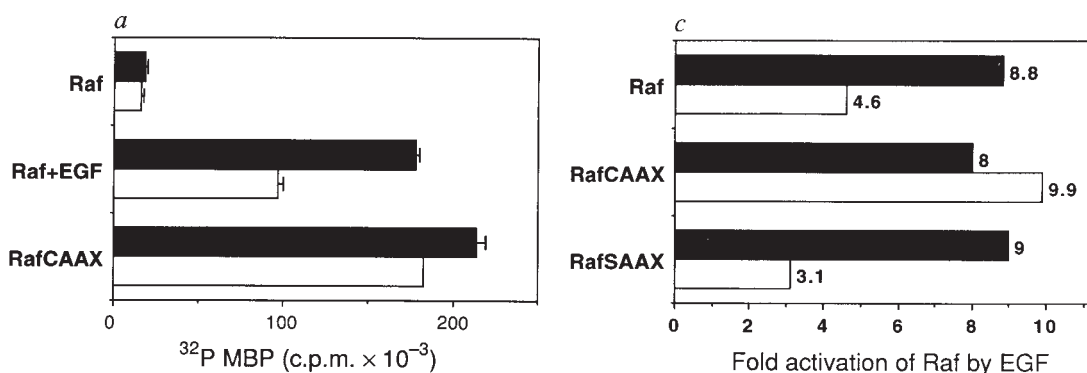


FIG. 4 Raf-CAAX activity is independent of cellular Ras. *a*, Kinase activity of Raf-CAAX is not inhibited by dominant-negative N17Ras. Lysates prepared from COS-1 cells co-transfected with vectors encoding Raf or Raf-CAAX, and dominant negative N17Ras<sup>25</sup> (open bars), or an inactive version of that protein (N17S186Ras; solid bars) were assayed for Raf activity as for Fig. 3. Treatment with 10  $\text{ng ml}^{-1}$  EGF was for 10 min; results are the mean (+average error from mean) of duplicate determinations after subtraction of the assay background, determined by assaying lysates of COS-1 cells transfected with empty vector(s). *b*, Raf-CAAX-induced PC12 cell neurite formation is not inhibited by N17Ras. Cells were stained with 9E10 (ref. 11) to reveal epitope-tagged Raf-CAAX (left panel) and with monoclonal antibody Y13-259 (ref. 28) to reveal N17Ras (right panel). Scale bar, 50  $\mu\text{m}$ . *c*, EGF-induced Raf-CAAX activation is not inhibited by N17-Ras expression. Lysates prepared from control and EGF-stimulated COS-1 cells co-transfected with vectors encoding Raf, Raf-CAAX or Raf-SAAX and dominant-negative N17Ras (open bars) or an inactive version of that protein (N17S186Ras; solid bars) were assayed for Raf activity as for Fig. 3, then the fold activation of Raf stimulated by EGF was calculated from the mean of duplicate determinations after subtraction of the assay background, determined by assaying lysates of COS-1 cells transfected with empty vector(s).

METHODS. PC12 cells were microinjected with a mixture of pmt-SM-Raf-CAAX (25  $\mu\text{g ml}^{-1}$ ) and pEXV3-N17Ras (100  $\mu\text{g ml}^{-1}$ ) DNAs. After 20 h, cells were fixed and double-labelled with 9E10 and Y13-238/259 antibodies and cells expressing both exogenous Raf and Ras were examined for neurite outgrowth.





sion system is achieved by co-expression of Ras and v-Src, suggesting that Ras cooperates with other signals to activate Raf. We obtained similar results in COS-1 cells and therefore investigated whether Raf-CAAX could be further activated by growth factors. EGF treatment induced an 8-fold increase in the kinase activity of Raf-CAAX, Raf-SAAX and Raf, but in contrast to Raf and Raf-SAAX, EGF-induced activation of Raf-CAAX was independent of cellular Ras (Fig. 4c).

Our results show that Raf translocates to the plasma membrane in the presence of Ras-GTP and that Raf targeted to

the plasma membrane by a Ras membrane-localization signal is constitutively activated independently of cellular Ras. Furthermore, the additional activation of membrane-targeted Raf following growth factor stimulation is also independent of cellular Ras. These data are consistent with Ras functioning as an activatable membrane-bound anchor for Raf. It remains to be discovered what takes place at the plasma membrane to activate Raf, although protein phosphorylation or the interaction of Raf with another molecule such as lipid are plausible possibilities<sup>18,21,23</sup>. □

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- Marshall, C. J. *Curr. Opin. Gen. Dev.* **4**, 82–89 (1994).
- Dickson, B. & Hafen, E. *Curr. Opin. Gen. Dev.* **4**, 64–70 (1994).
- Van Aelst, L., Barr, M., Marcus, S., Polverino, A. & Wigler, M. *Proc. natn. Acad. Sci. U.S.A.* **90**, 6213–6217 (1993).
- Votjek, A. B., Hollenberg, S. M. & Cooper, J. A. *Cell* **74**, 205–214 (1993).
- Warne, P. H., Rodriguez Viciano, P. & Downward, J. *Nature* **364**, 352–355 (1993).
- Zhang, X.-f. et al. *Nature* **364**, 308–313 (1993).
- Moodie, S. A., Willumsen, B. M., Weber, M. J. & Wolfman, A. *Science* **260**, 1658–1661 (1993).
- Koide, H., Satoh, T., Nakafuku, M. & Kaziro, Y. *Proc. natn. Acad. Sci. U.S.A.* **90**, 8683–8686 (1993).
- Finney, R. E., Robbins, S. M. & Bishop, J. M. *Curr. Biol.* **3**, 805–811 (1993).
- Hallberg, B., Rayter, S. I. & Downward, J. *J. biol. Chem.* **269**, 3913–3916 (1994).
- Evan, G. I., Lewis, G. K., Ramsay, G. & Bishop, J. M. *Molec. cell. Biol.* **5**, 3610–3616 (1985).
- Schultz, A. M., Copeland, T. D., Mark, G. E., Rapp, U. R. & Oroszlan, S. *Virology* **146**, 78–89 (1985).
- Force, T. et al. *Proc. natn. Acad. Sci. U.S.A.* **91**, 1270–1274 (1994).
- Traverse, S. et al. *Oncogene* **8**, 3175–3181 (1993).
- Hancock, J. F., Magee, A. I., Childs, J. E. & Marshall, C. J. *Cell* **57**, 1167–1177 (1989).

- Hancock, J. F., Paterson, H. & Marshall, C. J. *Cell* **63**, 133–139 (1990).
- Leevers, S. J. & Marshall, C. J. *EMBO J.* **11**, 569–574 (1992).
- Bruder, J. T., Heidecker, G. & Rapp, U. *Genes Dev.* **6**, 545–556 (1992).
- Feig, L. A. & Cooper, G. M. *Molec. cell. Biol.* **8**, 3235–3243 (1988).
- Williams, N. G., Roberts, T. M. & Li, P. *Proc. natn. Acad. Sci. U.S.A.* **89**, 2922–2926 (1992).
- Fabian, J. R., Daar, I. O. & Morrison, D. K. *Molec. cell. Biol.* **13**, 7170–7179 (1993).
- Sözeri, O. et al. *Oncogene* **7**, 2259–2262 (1992).
- Kolch, W. et al. *Nature* **364**, 249–252 (1993).
- Gómez, N. & Cohen, P. *Nature* **353**, 170–173 (1991).
- Howe, L. R. et al. *Cell* **71**, 335–342 (1992).
- Stokoe, D. et al. *EMBO J.* **11**, 3985–3994 (1992).
- Alessi, D. R. et al. *EMBO J.* **13**, 1610–1619 (1994).
- Furth, M. E., Davis, L. J., Fleurdelys, B. & Scolnick, E. M. *J. Virol.* **43**, 294–304 (1982).
- Schaap, D., van der Wal, J., Howe, L. R., Marshall, C. J. & van Blitterswijk, W. J. *J. biol. Chem.* **268**, 20232–20236 (1993).

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## A second signal supplied by insulin-like growth factor II in oncogene-induced tumorigenesis

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TRANSGENIC mice expressing the simian virus-40 large T-antigen (Tag) under the control of the insulin gene regulatory region offer a useful model for tumorigenesis<sup>1,2</sup>. All the islets of Langerhans express Tag, although there is at first no aberrant proliferation. Over half of the islets become hyperplastic, however, and neovascularization of a further subset (about 10%)<sup>3</sup> leads eventually to formation of highly vascularized solid tumours in 1–2% of islets by about 14 weeks of age. Here we show that the initial proliferative switch is correlated with focal activation of insulin-like growth factor II (IGF-II). Transfection with an antisense oligonucleotide to the IGF-II messenger RNA interferes with tumour cell proliferation *in vitro*, and transgenic mice homozygous for a disruption of the IGF-II gene develop tumours with reduced malignancy and a higher incidence of apoptosis. Several signals, in this case including an oncoprotein and a growth/survival factor, thus appear to be needed to elicit hyperproliferation.

To assess their role in oncogenesis, we compared the expression of about 30 growth factors, receptors and oncogenes in normal pancreatic islets and the  $\beta$ -cell tumours from several lines of RIP-Tag transgenic mice. IGF-II is widely expressed in the developing mouse embryo, but its expression is progressively extinguished in virtually all tissues after birth<sup>4,6</sup>. IGF-II was, however, selectively upregulated in the tumours. In contrast, the

low levels of IGF-I mRNA in normal islets were further reduced after the onset of tumorigenesis; the levels of both types of IGF receptor were unchanged at all stages of tumour formation. Radioreceptor competition assays showed that cultured tumour cells express functional IGF-I and IGF-II receptors and release IGF-II into conditioned medium (data not shown).

Sporadic hyperproliferation of the ~400 islets expressing the Tag oncoprotein begins at about 3–4 weeks<sup>2</sup>. *In situ* RNA hybridization showed that IGF-II was expressed in the pancreas of 12–14-day-old juvenile, non-transgenic mice (Fig. 1a), but the core of the islets showed little or no signal, indicating that IGF-II is either not normally expressed or has ceased to be expressed there by this time. IGF-II mRNA could not be detected anywhere in the pancreas of adult (>4 weeks old) control mice, including the islets (Fig. 1d), consistent with RNA-polymerase chain reaction (PCR) results (not shown). IGF-II expression in the pancreas of 12–14-day transgenic mice was similar, although an occasional islet showed a clear IGF-II signal in the core where  $\beta$ -cells are located (not shown). By 4 weeks, IGF-II was undetectable throughout the exocrine pancreas of transgenic mice, and many islets did not express IGF-II (Fig. 1b). Other islets were positive (Fig. 1c, e), however, as was every tumour that developed by 12–14 weeks (Fig. 1f). An RNA-PCR analysis of single transgenic islets confirmed that only a fraction expressed IGF-II (not shown). In some islets, IGF-II expression was clearly localized (Fig. 1c, e), whereas in larger hyperplastic islets the pattern was more uniform (Fig. 1c). IGF-II is thus focally activated in a subset of the islets expressing Tag and is further upregulated in all tumours.

IGF-II expression in pancreatic sections from RIP-Tag2 mice at different ages and stages of tumour development was analysed by *in situ* hybridization, and hyperproliferation was assessed by immunostaining with antibodies against PCNA, a subunit of DNA polymerase  $\delta$  expressed in the late G1 and S phases of proliferating cells<sup>7,8</sup>. Some adjacent sections were also immunostained to detect Tag. Hyperproliferation and IGF-II expression were clearly correlated: islets with extensive PCNA staining had high levels of IGF-II mRNA (Fig. 2A, d–f). In contrast, Tag was expressed in all islets, including those with no detectable IGF-II mRNA and low PCNA expression (Fig. 2A, a–c). Some islets with focal expression of IGF-II mRNA (such as Fig. 1e)

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FIG. 1 IGF-II expression is focally activated in hyperplastic islets and further upregulated in tumours. *In situ* hybridization analysis using a  $^{35}\text{S}$ -labelled IGF-II probe was done on tissue sections of RIP-Tag2 transgenic and C57Bl/6J control mice at varying ages. Dark-field illumination micrographs are shown. *a*, IGF-II expression in the pancreas of a 12-day-old juvenile mouse; the islet core of  $\beta$  cells shows little or no signal. *d*, Islet in a 6-week-old control mouse, which does not express detectable IGF-II. The remaining panels illustrate the major classes of IGF-II expression seen in islets from 4–14-week-old RIP1-Tag2 transgenic mice: *b*, a non-expressing islet; *e*, an islet with focal expression; *c*, a pair of islets, one a hyperplastic islet uniformly expressing IGF-II, and the second expressing in a weaker, more focal pattern; and *f*, a tumour expressing high levels of IGF-II mRNA. Arrows indicate islets with low or no detectable IGF-II expression. Magnification in *f*,  $\times 25$ ; in *a–e*,  $\times 50$ .

**METHODS.** *In situ* hybridization on mouse pancreas tissues was as described<sup>24</sup> with some modifications. Briefly, after perfusion fixation with 4% paraformaldehyde, the pancreas was removed and post-fixed in 4% paraformaldehyde. 10- $\mu\text{m}$  cryosections were prepared for *in situ* hybridization.  $^{35}\text{S}$ -labelled sense and antisense RNA probes were synthesized from a linearized subclone (insert fragment spanning the entire mouse IGF-II coding region, including 34 nucleotides upstream of the start codon and 858 nucleotides downstream of the stop codon, ligated into pBluescript), using

the T3 or T7 polymerase, respectively. Cryosections were treated with proteinase K (5  $\text{mg ml}^{-1}$  for 10 min), acetylated with acetic anhydride (10 min), and hybridized with RNA probes (600,000 c.p.m. per section) at 55 °C overnight in hybridization solution. Following hybridization, sections were washed and then treated with RNase A (20  $\text{mg ml}^{-1}$  for 30 min), followed by a wash in 0.1  $\times$  SSC/10 mM  $\beta$ -mercaptoethanol (55–58 °C for 2 h). Vacuum-dried slides were dipped in Kodak NTB2 emulsion, and developed a week later followed by counterstaining with haematoxylin–eosin.

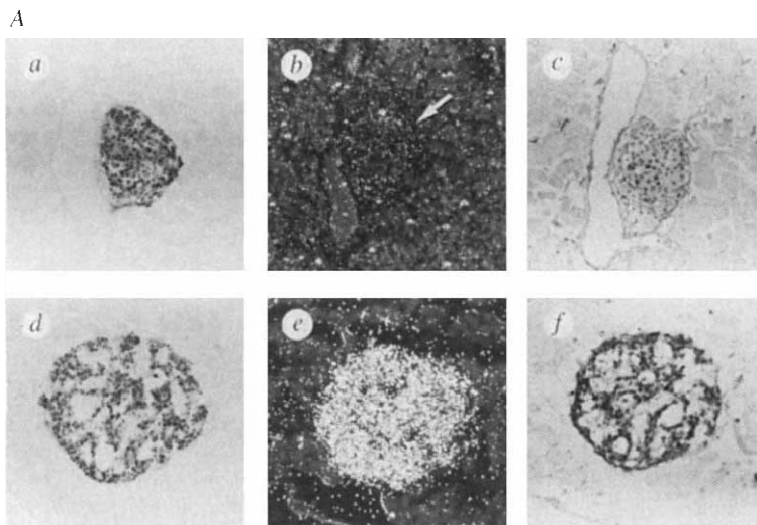
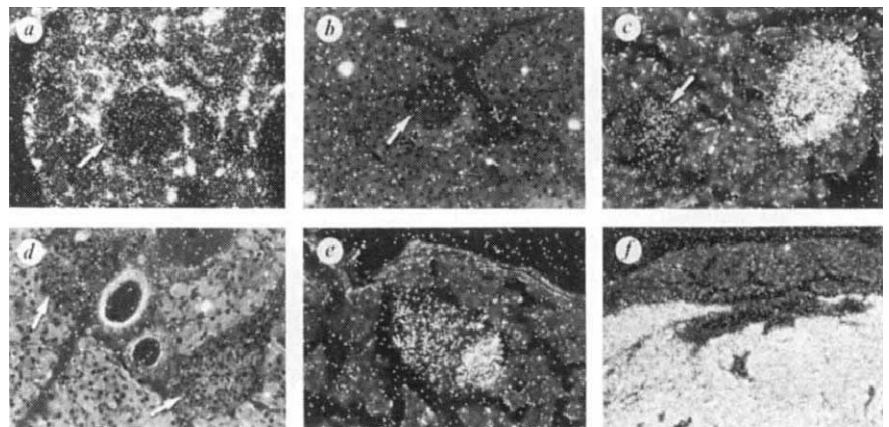
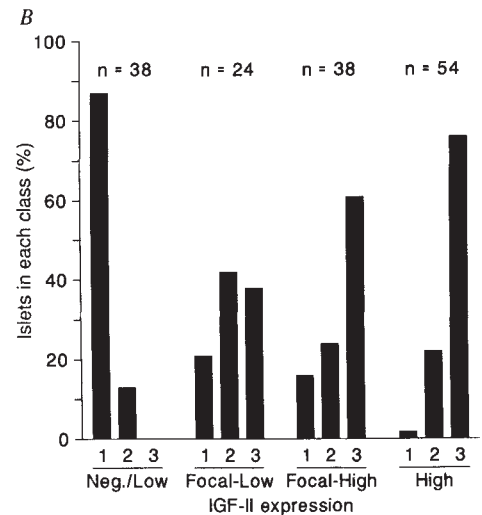


FIG. 2 Activation of IGF-II correlates with the switch to hyperproliferation. *A*, IGF-II expression coincides with that of PCNA, a marker of proliferating cells. *a–c*, Transgenic islet that expressed the Tag oncoprotein (*a*) but not IGF-II (*b*, arrow); this islet is not hyperplastic, as indicated by the low number of nuclei intensely staining for PCNA (*c*). *d–f*, Serial sections of a hyperplastic islet immunostained for the Tag oncoprotein (*d*), and for PCNA (*f*), and analysed for IGF-II expression by *in situ* hybridization (*e*). Only nuclei strongly reactive for the anti-PCNA antibody represent proliferating cells, as determined by comparison with non-proliferating cells in non-transgenic islets. Note that both islets contain  $\beta$  cells expressing the Tag oncoprotein at similar levels. Brightfield (*a*, *c*, *d*, *f*) and dark-field (*b*, *e*) illumination photomicrographs are shown. Magnification,  $\times 50$ . *B*, Statistical analysis of the correlation between IGF-II activation and the switch to the hyperproliferative stage. Four classes of islets were identified in analysis of tissue sections of RIP1-Tag2 transgenic mice: islets with no detectable IGF-II mRNA (Neg/Low); islets with low focal IGF-II expression (Focal/Low); islets with high focal IGF-II expression (Focal/High); and islets uniformly positive for IGF-II (High). Altogether 154 islets were analysed by IGF-II *in situ* hybridization and PCNA immunostaining of adjacent tissue sections from 6 transgenic mice at ages varying from 4 weeks to 14 weeks. Each class of islet was given a score for its proliferation index, as assessed by PCNA staining. The data are summarized as the fractions of each IGF-II expression class that show a distinctive proliferation (PCNA) index: (1) off/low, 0–15%; (2) medium, 15–25%; or (3) high, >25% PCNA-positive cells.

**METHODS.** *A*, *In situ* hybridizations were as described in the legend to Fig. 1. For Tag staining, tissue sections were rehydrated in PBS and then incubated in 5%  $\text{H}_2\text{O}_2$ , methanol (20 min). Retrieval of the antigen was achieved by microwaving the tissue sections twice for 5 min in antigen retrieval Citra



solution (Biogenex, San Ramon, CA). The tissues were then permeabilized in PBS/0.25% Nonidet P40 (15 min), followed by blocking in 10% goat serum (30 min). A rabbit polyclonal antibody specific against Tag was placed on the tissue sections at a dilution of 1:1,000 in PBS, 1% goat serum (overnight). After three washes in PBS, 1% goat serum, a peroxidase-conjugated goat anti-rabbit IgG was applied at 1:200 in PBS, 1% goat serum for 45 min. The sections were then washed three times in PBS, 1% goat serum and the immunocomplexes visualized by treatment with diaminobenzidine, nickel sulphate and  $\text{H}_2\text{O}_2$ . After brief washes in PBS and dehydration through graded alcohol, the sections were mounted in Entellan New mounting medium (EM Science, Gibbstown, NJ). For PCNA staining, rehydration of tissue sections, blocking of endogenous peroxidase and antigen retrieval were as for Tag staining. Sections were blocked in PBS, 10% goat serum, 0.3% Triton X-100 (30 min). After a brief wash in PBS, a mouse monoclonal antibody against PCNA (Biogenex, San Ramon, CA) was applied at 1:100 dilution in PBS, 2% goat serum, 0.3% Triton X-100 (2 h). After three washes in PBS, a biotinylated goat-anti-mouse IgM was placed on the sections at a 1:200 dilution in PBS, 2% goat serum, 0.3% Triton X-100 (45 min). Again, after three washes in PBS, Vectastain Elite ABC reagent was applied (45 min). The sections were then washed and the avidin–biotin immunocomplexes visualized by treatment with diaminobenzidine, nickel sulphate and  $\text{H}_2\text{O}_2$ . Dehydration and mounting were as for T-antigen staining. *B*, The PCNA proliferation index was determined by calculating the number of PCNA-positive nuclei as a percentage of the total number of nuclei in individual islets. The statistical analysis was done by assigning the proliferation index of a particular islet to one of the four classes of IGF-II expression, as revealed by *in situ* hybridization analysis of the same islet on an adjacent tissue section.



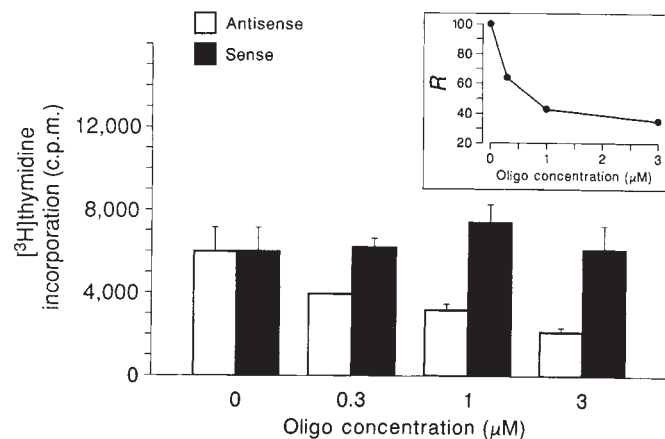


FIG. 3 Interference with IGF-II attenuates proliferation of  $\beta$  tumour cells in culture. Sense and antisense oligonucleotides to IGF-II mRNA were added in increasing concentrations to  $\beta$ TC3 cells, and the incorporation of tritiated thymidine was monitored. The inset gives the ratio (R) of antisense to sense oligonucleotides, demonstrating the specificity of the antisense inhibition. Phosphothiol-linked oligonucleotides are shown. Traditional phosphodiester-linked oligos gave a similar, although less effective, inhibition by the antisense oligo. Additional controls included antisense oligos complementary to mRNAs of three other growth factors expressed in the tumours: IGF-I, the endothelial mitogen VEGF, and betacellulin (not shown).

**METHODS.**  $\beta$ TC3 were seeded at  $1 \times 10^5$  cells per well in 48-well plates and grown in DME-H21 plus 10% fetal calf serum (FCS) to a confluency of 30 to 50%. Medium was changed to DME-H21 plus 2% FCS 6 h before oligos were added at varying dilutions in PBS as indicated. After 18 h,  $1 \mu$ Ci tritiated thymidine was added per well and the cells were incubated for an additional 6 h. Non-incorporated thymidine was removed by washing with PBS, and the cells were processed for the determination of thymidine incorporation by scintillation counting: washes in methanol ( $2 \times 5$  min), 5% trichloroacetic acid ( $2 \times 10$  min), water ( $1 \times 1$  min). The DNA was resuspended in 0.3 M sodium hydroxide and added to 3 ml scintillation fluid and counted. Oligodeoxynucleotide sequences were antisense IGF-II: GTACCCACTGGGATCCCA and sense IGF-II: TGGGGATCCCACTGGGGTAC<sup>15</sup>.

showed more localized PCNA staining (not shown), indicating that IGF-II release can induce local cell proliferation. Simultaneous visualization of both markers in the same tissue section was not possible, but a statistical analysis of serial sections confirmed the correlation between hyperproliferation and IGF-II expression (Fig. 2B). IGF-II is thus activated in islets in which the  $\beta$ -cells have begun to proliferate, consistent with its involvement in this step of tumorigenesis.

Oligodeoxynucleotides (oligos) bearing sense and antisense sequences derived from IGF-II message were transfected into cultured tumour cells ( $\beta$ TC3 cell line)<sup>9</sup>, and the relative number of cells in S phase was monitored by incorporation of tritiated thymidine. The antisense strand attenuated thymidine incorporation in a dose-dependent manner, whereas the sense control oligo had no effect (Fig. 3). Analogous experiments with genes for three other growth factors expressed in the  $\beta$ -cell tumours did not interfere with  $\beta$ -cell proliferation; these were IGF-I, VEGF, which encodes an endothelial cell-specific mitogen (G.C. *et al.*, manuscript in preparation) and betacellulin, an EGF family member first isolated from the  $\beta$ TC lines<sup>10</sup>. IGF-II thus appears to be a component of the growth signalling pathway for  $\beta$  tumour cells.

To examine the contribution of IGF-II to  $\beta$ -cell tumorigenesis further, the RIP1-Tag2 transgenic mice were crossed into a line of mice in which the IGF-II gene was disrupted<sup>11,12</sup>. Remarkably, IGF-II<sup>-/-</sup> mice develop normally, are fertile, and have typical longevity, although their body mass is reduced by about 40% (refs 11, 12). In comparison to age-matched RIP1-Tag2 IGF-II<sup>+/+</sup> mice, RIP1-Tag2 IGF-II<sup>-/-</sup> mice developed fewer

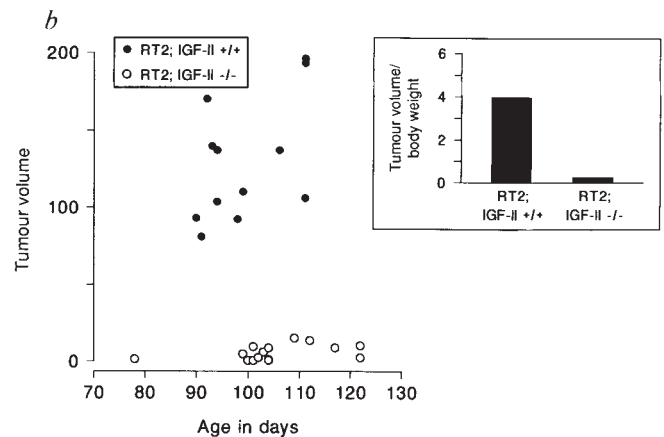
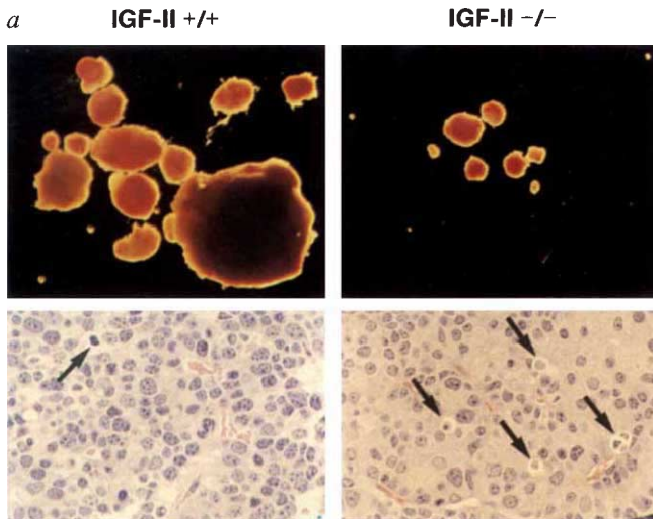
FIG. 4 Complementation analysis with IGF-II null mice results in impaired tumour growth. **a**, Gross pathology (top) and histopathology (bottom). Top, tumours that arose in a single RIP1-Tag2 mouse that was either wild-type or deficient in IGF-II are shown at  $\times 6.5$ . Fewer, and obviously smaller tumours arose in the IGF-II<sup>-/-</sup> mouse. Bottom, semithin tissue sections of tumours from RIP1-Tag2, IGF-II<sup>+/+</sup> or IGF-II<sup>-/-</sup> mice were stained with haematoxylin and eosin. The IGF-II null tumour cells are less densely packed, and have smaller, more normal nuclei, and larger cytoplasm. The tumours in the IGF-II null mice have more apoptotic cells than their IGF-II<sup>+/+</sup> counterparts (arrows, apoptotic bodies). Magnification,  $\times 50$ . To quantify the incidence of apoptosis, histological sections of 10 tumours from RIP1-Tag2, IGF-II<sup>+/+</sup> and 7 from RIP1-Tag2, IGF-II<sup>-/-</sup> mice were analysed for apoptotic cells in 210 and 119 fields, respectively, revealing  $0.88 \pm 0.84$  and  $4.50 \pm 1.63$  apoptotic cells per field in tumours from RIP1-Tag2, IGF-II<sup>+/+</sup> and RIP1-Tag2, IGF-II<sup>-/-</sup>, respectively, indicating a 5.2-fold increase in apoptosis ( $P < 0.001$ ; Student's *t*-test). **b**, Comparison of tumour volume between IGF-II proficient and IGF-II null RIP1-Tag2 transgenic mice. A histogram shows the large reduction in tumour volume at autopsy in a series of individual RIP1-Tag2, IGF-II<sup>-/-</sup> mice, compared to RIP1-Tag2, IGF-II<sup>+/+</sup> littermates. Inset compares average tumour volumes, corrected for body weight; body weight for the IGF-II null mice is typically 60% of age-matched controls. Serum glucose levels suggest that the small tumours in the IGF-II null mice are sufficient to elicit lethal hypoglycaemia. **c**, A model: two signals, both from an oncogene and confirmatory growth/survival factors, are necessary to elicit hyperproliferation. Analysis of the focal switch to hyperproliferation, after activation of the Tag oncogene, and concomitant with upregulation of IGF-II, suggests that two signals are necessary to instruct a normal  $\beta$ -cell in its natural environment to proliferate. The Tag oncogene provides one of these, and IGF-II is a component of the second signal. In the absence of a confirmatory second signal, expression of the oncogene may result in continued quiescence (as seen here), or induction of apoptosis, as revealed by experiments with conditional oncogenes in tissue culture<sup>21,22</sup>. Tumours still develop in the IGF-II null mice, indicating there is another component (factor 'x') to the second signal. However, this putative factor 'x' is not sufficient to induce a high-grade malignancy in the absence of IGF-II; the Tag-expressing cells are only partially transformed, resulting in more frequent apoptosis and impaired tumour growth.

**METHODS.** **a**, Mice were killed by cervical dislocation and pancreas and tumours removed and immersion-fixed for 16 h in 4% paraformaldehyde, PBS at 4 °C. Tissues were immersed at 4 °C three times in one

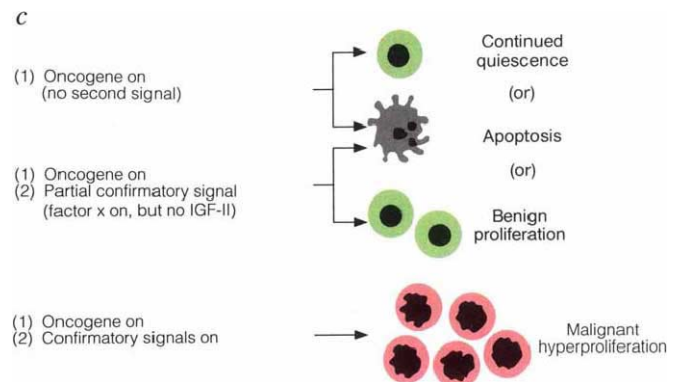
tumours of dramatically reduced size (Fig. 4a). The tumour cells in the IGF-II null mice were less densely packed, with smaller nuclei and more cytoplasm than those in mice with functional IGF-II, indicating that they represent a lower-grade malignancy. Remarkably, the number of apoptotic cell bodies (see arrows in Fig. 4a) in the IGF-II null tumours was fivefold higher (for details see Fig. 4 legend); the two classes of mice are compared in terms of lifespan and total tumour volume at autopsy in Fig. 4b. The average volume of the tumours in RIP1-Tag2, IGF-II<sup>-/-</sup> is about 7% of that in their IGF-II<sup>+/+</sup> counterparts (corrected for body weight). Although these small tumours are eventually lethal, probably as a result of hypoglycaemia elicited by the insulin-producing  $\beta$ -cells, IGF-II clearly contributes to tumour growth and malignancy *in vivo*.

Hyperproliferation preceding tumour formation is statistically correlated with IGF-II expression, and reduced IGF-II expression impairs tumour cell growth *in vitro* and *in vivo*. IGF-II thus appears to be part of a rate-limiting step in multistage oncogenesis, promoting tumour growth and malignancy, consistent with IGF-I or IGF-II expression in a variety of tumour types *in vitro* (reviewed in ref. 13). The way in which its expression is upregulated is unclear; a preliminary analysis indicates that the gene is not rearranged or amplified. Although genomic imprinting restricts expression of the maternal allele to the brain in wild-type mice<sup>11,14,15</sup>, both alleles are activated during the switch to hyperproliferation in the oncogene-expressing  $\beta$ -cells (G.C., P.N. and D.H., manuscript submitted).

Transgenic mouse models of tumorigenesis have shown that tumours arise through a series of distinguishable stages and that



► hour in 0.1 M phosphate buffer, pH 7.4, 50 mM ammonium chloride, dipped three times for 10 min in cold acetone before being embedded in JB4 plastic medium according to the manufacturer's protocol (Polysciences, Warrington, PA). Sections were cut at 1.5  $\mu$ m on a BioRad microtome (BioRad, Richmond, CA) using a glass knife and stained with haematoxylin, eosin and azure-blue. Apoptotic cells were identified by their pyknotic nuclei and detachment from neighbouring cells. *b*, Mice were killed by cervical dislocation at ages as indicated, their body weight was determined and tumours were isolated either by retrograde perfusion with collagenase solution<sup>25</sup> or by microdissection. The number of tumours and their diameters were determined and the total tumour volume was calculated as a function of a roughly spherical tumour shape. Tumour volumes were plotted as a function of age. The difference between the two groups was statistically significant ( $P < 0.001$ ; Student's *t*-test). In addition, the ratio of the average tumour volume to the average body weight of 9 RIP1-Tag2: IGF-II<sup>+/+</sup> and 12 RIP1-Tag2: IGF-II<sup>-/-</sup> mice was determined (see inset).



only a small fraction of oncogene-expressing cells develop into tumours (reviewed in refs 16–18). Similarly, although Tag is expressed in all  $\beta$ -cells of the transgenic mice from embryonic day 9 (ref. 19),  $\beta$ -cell hyperproliferation is first evident only 3–4 weeks later<sup>2</sup>. In addition to the oncogene, a second signal is apparently needed for proliferation, of which IGF-II is part. The small tumours in IGF-II null mice indicate that they still express another component of this signal, albeit one inadequate to induce hyperproliferation and full malignancy; its identity will be of interest.

Several lines of evidence suggest there are two alternative responses to oncogene expression when the second signal is absent or incomplete: continued quiescence, apoptosis, or benign proliferation (Fig. 4c). The proliferation index of premalignant  $\beta$ -cells is similar with and without IGF-II (data not shown), but the incidence of apoptotic cells is increased from 0.5 to 2.5%. In the rat optic nerve, half the 20,000 oligodendrocytes produced each day die, although the incidence of apoptotic cells seen in histological sections is only 0.25% (ref. 20); thus the fivefold increase in apoptotic index in IGF-II null mice represents a significant change that may well account for their reduced tumour mass. In cultured cells expressing the oncogene *c-myc*, serum growth factors are necessary to prevent apoptosis<sup>21</sup>; IGF-II, IGF-I and PDGF have all been shown to be active in this assay<sup>22</sup>. The effects of IGF-II on tumour growth may therefore reflect its role as a survival factor. The markedly increased malignancy seen in the IGF-II (+/+) tumours, however, indicates that it has additional effects on the tumour cell phenotype.

Continued quiescence or apoptosis of oncogene-expressing cells in the absence of further signals indicates that at least two

signals are needed to initiate proliferation of fully differentiated cells (much as multiple signals are needed to activate the immune system<sup>23</sup>), thereby minimizing the possibility that mere upregulation of an oncogene could elicit hyperproliferation (with the danger of progression to neoplasia). IGF-II is evidently a key component of the second signal in the model of tumorigenesis studied here, and the frequent reports of its expression in other tumours suggest that it may often serve this role elsewhere. □

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1. Hanahan, D. *Nature* **315**, 115–122 (1985).
2. Teitelman, G., Alpert, S. & Hanahan, D. *Cell* **52**, 97–105 (1988).
3. Folkman, J. et al. *Nature* **339**, 58–61 (1989).
4. Rechler, M. M. & Nissley, S. P. in *Peptide Growth Factors and their Receptors I: Handbook of Experimental Pharmacology* Vol. 95 (eds Sporn, M. B. & Roberts, A. B.) 263–367 (Springer, Berlin, New York, 1990).
5. LeRoith, D. (ed.) *Insulin-like Growth Factors: Molecular and Cellular Aspects* (CRC Press, Boca Raton, 1991).
6. Schofield, P. N. (ed.) *The Insulin-like Growth Factors: Structure and Biological Function* (Oxford Univ. Press, Oxford, 1992).
7. Garcia, R. L. et al. *Am. J. Path.* **134**, 733–739 (1989).
8. Bravo, R. et al. *Nature* **326**, 515–517 (1987).
9. Efrat, S. et al. *Proc. natn. Acad. Sci. U.S.A.* **85**, 9037–9041 (1988).
10. Shing, Y. et al. *Science* **259**, 1604–1607 (1993).
11. DeChiara, T. M., Robertson, E. J. & Efstratiadis, A. *Cell* **64**, 849–859 (1991).
12. DeChiara, T. M., Efstratiadis, A. & Robertson, E. J. *Nature* **345**, 75–80 (1990).
13. Schofield, P. N. & Engstrom, W. in *The Insulin-like Growth Factors: Structure and Biological Function* (ed. Schofield, P. N.) (Oxford Univ. Press, Oxford, 1992).
14. Ferguson-Smith, A. C. et al. *Nature* **351**, 155–159 (1991).
15. Rappolee, D. A. et al. *Genes Dev.* **6**, 939–952 (1992).
16. Hanahan, D. *Science* **246**, 1265–1275 (1989).
17. Hanahan, D. *Rev. Genet.* **22**, 479–519 (1988).
18. Adams, J. & Cory, S. *Science* **254**, 1161–1167 (1991).
19. Alpert, S., Hanahan, D. & Teitelman, G. *Cell* **53**, 295–308 (1988).
20. Barres, B. A. et al. *Cell* **70**, 31–46 (1992).
21. Evan, G. I. et al. *Cell* **69**, 119–128 (1992).
22. Harrington, E. A., Bennett, M. R., Faniadi, A. & Evan, G. I. *EMBO J.* (in the press).
23. Bretscher, P. & Cohn, M. *Science* **167**, 1042–1049 (1970).



24. Smith, K. M., Lawn R. M. & Wilcox, J. N. *J. Lipid Res.* **31**, 995–1004 (1990).  
 25. Lacy, P. E. & Kostianovsky, M. *Diabetes* **16**, 35–39 (1967).

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## Ras-dependent activation of MAP kinase pathway mediated by G-protein $\beta\gamma$ subunits

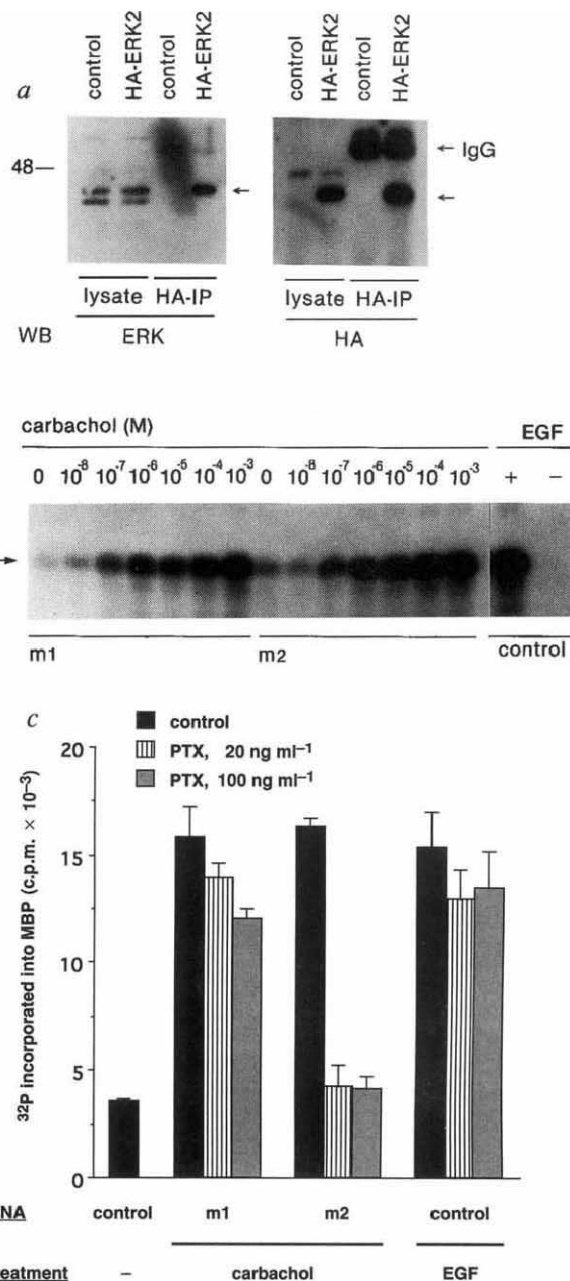
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**MITOGEN-ACTIVATED protein kinases, MAP kinases or ERKs (extracellular signal-regulated kinases) are rapidly stimulated by growth-promoting factors acting on a variety of cell-surface receptors<sup>1,2</sup>. In turn, ERKs phosphorylate and regulate key intracellular enzymes and transcription factors involved in the control of cellular proliferation<sup>3,4</sup>. The tyrosine-kinase class of growth-factor receptors transmits signals to ERKs in a multistep process that involves Ras and a limited number of defined molecules<sup>5</sup>. In contrast, ERK activation by G-protein-coupled receptors is poorly understood<sup>3,6</sup>, as is the role of *ras* in this signalling pathway<sup>7,8</sup>. We have explored in COS-7 cells the mechanism of ERKs activation by m1 and m2 muscarinic receptors, typical examples of receptors coupled through Gq proteins to induce phosphatidylinositol hydrolysis and to  $G_i$  proteins to inhibit adenylyl cyclase, respectively<sup>9</sup>. Here we present evidence that ERK activation is mediated by  $\beta\gamma$  subunits of heterotrimeric G proteins acting on a *ras*-dependent pathway.**

Receptors m1 and m2 were expressed in COS-7 cells together with an epitope-tagged-ERK2 (HA-ERK2)<sup>10</sup> (Fig. 1a). In cells expressing either muscarinic receptor the cholinergic agonist carbachol induced a dose-dependent increase in HA-ERK2 activity (Fig. 1b). Treatment with pertussis toxin (PTX), which uncouples certain G proteins by ADP-ribosylation<sup>11</sup>, nearly abolished ERK2 activation mediated by m2 receptors, but not by m1 or EGF receptors (Fig. 1c). Thus, activation of ERK2 through m2 but not m1 or EGF receptors involves a PTX-sensitive G protein, presumably a member of the  $G_i$  family.

To investigate how G proteins signal to ERKs, we coexpressed HA-ERK2 together with wild-type or GTPase-deficient mutants of  $\alpha$  subunits from  $G_q$ ,  $G_{12}$ , and  $G_{13}$ <sup>12–16</sup>. All transfected  $\alpha$  subunits were detectably expressed (Fig. 2a). Activated  $\alpha_q$  and  $\alpha_{12}$ , as expected, potently induced phosphatidylinositol hydrolysis or inhibited adenylyl cyclase, respectively (refs 6, 14, 15 and data not shown), mimicking the effect of m1 or m2 stimulation. However, cells expressing either wild-type or activated forms of these  $G\alpha$  subunits did not display significantly enhanced HA-ERK2 phosphorylating activity (Fig. 2b). Furthermore, ERK2 was not significantly activated on expression of GTPase-deficient mutants representing each of the four known classes of G protein  $\alpha$ -subunits, alone or in all possible combinations (Fig. 2b). In contrast, *v-ras* (ref. 17) effectively activated ERK2 under identical experimental conditions. Thus, molecules in addition to  $\alpha$  subunits of G proteins must be involved in stimulating ERK activity.



**FIG. 1** Cholinergic agonist, carbachol, increases the enzymatic activity of an epitope-tagged ERK2 when coexpressed with m1 or m2 muscarinic receptors in COS-7 cells. **a**, Lysates containing 40  $\mu$ g of protein from COS-7 cells transfected with the pCDNA I vector (control) or with an expression plasmid containing the epitope-tagged murine ERK2 cDNA<sup>10</sup> (provided by M. J. Weber) (HA-ERK2, 1  $\mu$ g per plate) were subjected to western blot (WB) analysis with a C-terminal anti-ERK serum (UBI; ERK), or with epitope-tag-specific 12CA5 antibody (Babco) and a secondary rabbit anti-mouse serum (Cappel; HA). Parallel samples containing about 800  $\mu$ g of cellular protein were first immunoprecipitated with the 12CA5 antibody (HA-IP). Bands were visualized with <sup>125</sup>I-Protein A. The protein product of the HA-ERK2 construct is indicated with an arrow. **b**, COS-7 cells transfected with vector (control), m1 or m2 were serum starved overnight, lysed on stimulation with the indicated concentrations of carbachol or EGF (100 ng ml<sup>-1</sup>) for 5 min, and immunoprecipitated with the 12CA5 antibody (2  $\mu$ g). ERK kinase activity was determined in immunoprecipitates in a reaction buffer containing 1  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP, 20  $\mu$ M ATP and 1.5 mg ml<sup>-1</sup> myelin basic protein (MBP; Sigma) for 20 min at 30 °C, essentially as described<sup>10</sup>. Phosphorylated MBP was visualized by autoradiography after PAGE. Similar results were obtained in 3 independent experiments. **c**, Effect of pertussis toxin (PTX) on the activation of ERK2 by agonist in control, m1 or m2 transfected COS-7 cells. Untreated cells or cells treated for 18 h with 20 or 100 ng ml<sup>-1</sup> PTX were exposed to vehicle alone (minus), carbachol (100  $\mu$ M) or EGF (100 ng ml<sup>-1</sup>) for 5 min, and processed as above. Data represent average  $\pm$  s.e.m. of radioactivity incorporated in MBP in triplicate samples. Similar results were obtained in 3 independent experiments.

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When activated, receptors coupled to G proteins catalyse the replacement of GDP by GTP bound to the  $\alpha$  subunit. This exchange induces dissociation of  $\alpha$ -GTP from  $\beta\gamma$  dimers<sup>12,13,18</sup>. To investigate whether  $\beta\gamma$  complexes participate in the activation of ERKs, we took advantage of the finding that overexpression of the  $\alpha$  subunit of transducin ( $\alpha_t$ ) blocks  $\beta\gamma$ -dependent pathways, probably by sequestering free  $\beta\gamma$  dimers<sup>19</sup>. As shown in Fig. 3a, expression of  $\alpha_t$  did not affect the response of ERK2 to EGF but inhibited its activation when mediated by either muscarinic receptor, suggesting that  $\beta\gamma$  dimers play a role in signalling from G-protein-coupled receptors to ERKs.

We next determined whether overexpression of  $\beta\gamma$  subunits activates ERK2. In addition to the wild-type construct we used an altered  $\gamma$  subunit lacking an isoprenylation signal, designated  $\gamma^*$ , which dimerizes with  $\beta$  subunits but fails to associated with the plasma membrane<sup>20,21</sup>. Only cells cotransfected with both  $\beta$  and  $\gamma$  (or  $\gamma^*$ ) complementary DNAs expressed elevated levels of their respective protein products (Fig. 3b), probably because complex formation between  $\beta$  and  $\gamma$  is required for the stability of either G-protein subunit<sup>20-23</sup>. Coexpression of  $\beta\gamma$  but not of  $\beta\gamma^*$  led to a dramatic increase in ERK2 phosphorylating activity (Fig. 3c), demonstrating that  $\beta\gamma$ -heterodimers can directly

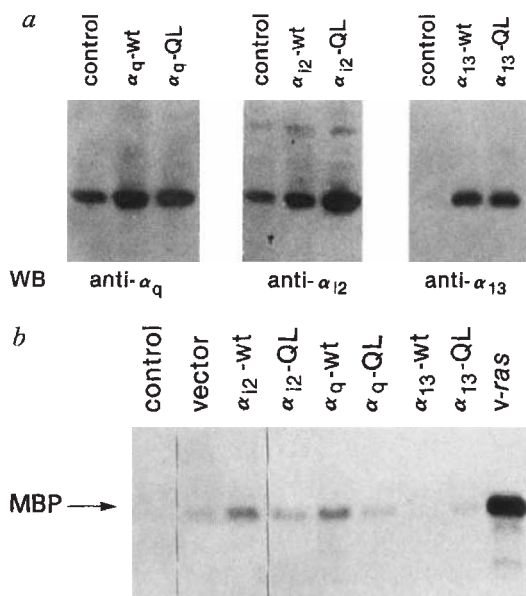


FIG. 2 Highly expressed wild-type or activated mutants of G-protein  $\alpha$  subunits fail to stimulate ERK2 enzymatic activity significantly. COS-7 cells were transfected with the pCDNA 1 vector (control) or the same vector carrying cDNAs for v-ras (ref. 17), wild-type murine  $\alpha_q$ ,  $\alpha_{12}$ ,  $\alpha_{13}$ , or for their corresponding activated mutants  $\alpha_q$  Q209L (ref. 15),  $\alpha_{12}$  Q207L (ref. 14) or  $\alpha_{13}$  Q226L (ref. 16 and N.X. et al., manuscript in preparation) (5  $\mu$ g per plate in each case), together with the HA-ERK2 expression plasmid (1  $\mu$ g per plate). a, Lysates containing 40  $\mu$ g total cellular proteins were subjected to western blot analysis using subunit specific antisera, as indicated. b, HA-ERK2 kinase activity was determined in anti-HA immunoprecipitates from the corresponding cellular lysates. Control cells were not transfected with HA-ERK2 plasmid. Small changes in  $^{32}$ P-labelled MBP in G-protein-transfected cells were not consistently observed, and therefore represent background variations. Similar results were obtained in 5 independent experiments. Similar lack of demonstrable ERK2-activation was observed on transfection of activated mutants of  $G_{\alpha_s}$  or  $G_{\alpha_{12}}$ , or when the different activated  $G_{\alpha}$  subunits were coexpressed in all possible combinations (data not shown).

FIG. 3 a,  $\alpha_t$  coexpression blocks agonist-induced activation of ERK2 in m1 or m2 transfected COS-7 cells. Plasmids containing m1 and m2 (1  $\mu$ g per plate) or the pCDNA 1 vector (control) were cotransfected with an expression vector carrying  $\alpha_t$  cDNA ( $\alpha_t$ , 5  $\mu$ g per plate) or with equal amount of vector. Cells were exposed to carbachol (100  $\mu$ M) or EGF (100 ng ml<sup>-1</sup>) for 5 min, lysed and ERK activity assayed on anti-HA immunoprecipitates as above. After autoradiography, gels were sliced and radioactivity incorporated in MBP counted. Data represent average  $\pm$  s.e.m. of triplicate samples. Similar results were obtained in 3 independent experiments. Inset, Cells transfected with the indicated plasmid DNA were analysed by western blotting with the C-terminal  $\alpha_t/\alpha$  antisera AS (ref. 20). The protein product of the transfected  $\alpha_t$  gene is indicated. The upper band present in both lysates represents endogenous  $\alpha_t$  proteins. b, c, Overexpression of G protein  $\beta$  and  $\gamma$  subunits stimulates ERK2 enzymatic activity. COS-7 cells were transfected with the pCDM8-1 vector (control) or the same vector carrying cDNAs for the  $\beta_1$  subunit, the wild-type  $\gamma_2$  subunit or an isoprenylation deficient mutant of  $\gamma_2$  ( $\gamma_2^*$ ) (5  $\mu$ g per plate in each case) together with pcDNA-HA-ERK2 plasmid (1  $\mu$ g per plate). Cotransfection of pcDNA-v-ras (5  $\mu$ g per plate) with the HA-ERK2-DNA was used as a control. b, 40  $\mu$ g total lysate proteins were subjected to western blot analysis using subunit specific antisera (C-terminal peptide  $\beta$  and  $\gamma_2$  antisera, SW and EDPL, respectively; W.S., manuscript in preparation), as indicated. c, HA-ERK2 kinase activity was determined in anti-HA immunoprecipitates obtained from cotransfected cells as shown. After autoradiography, gels were sliced and radioactivity incorporated in MBP counted. Data represent average  $\pm$  s.e.m. of triplicate samples. Similar results were obtained in 6 independent experiments.

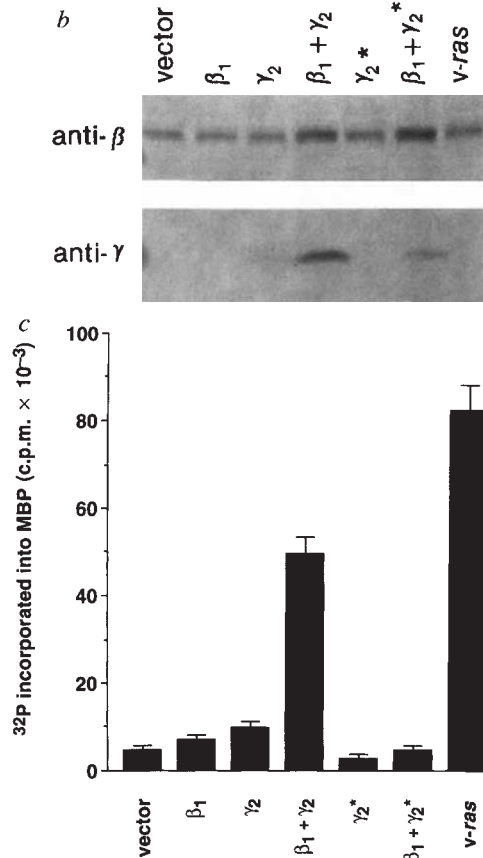
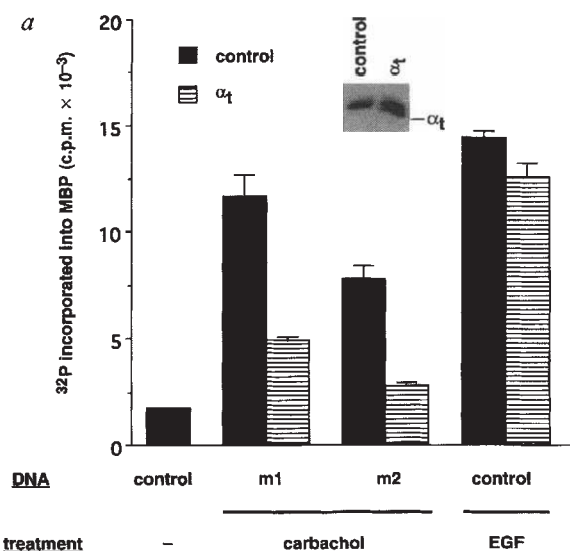
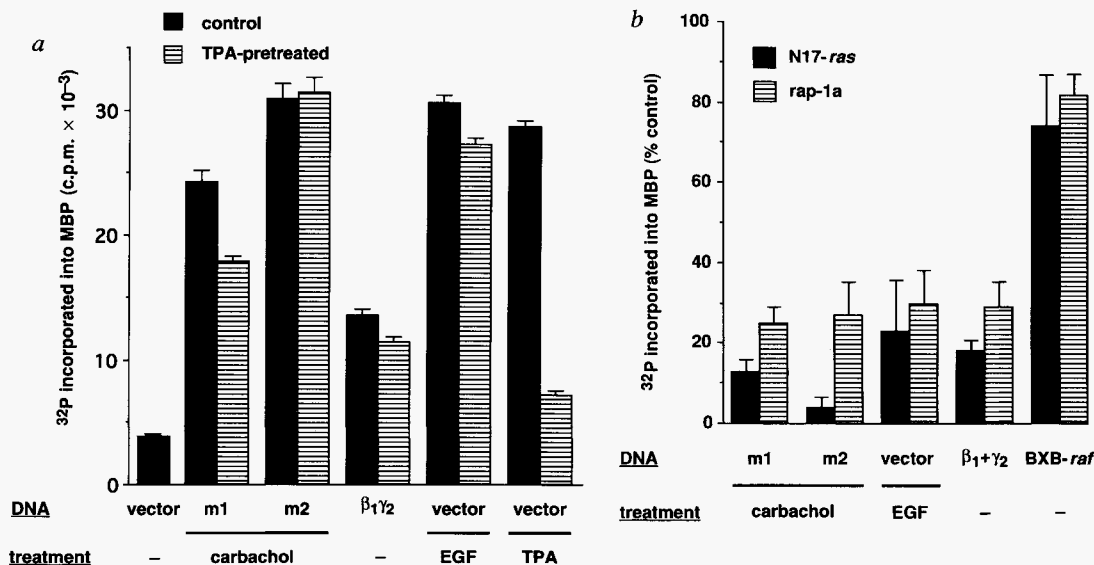




FIG. 4 a, Effect of protein kinase C downregulation on agonist or  $\beta\gamma$ -induced activation of ERK2. Cells were transfected with the indicated expression plasmids together with pcDNA-HA-ERK2. Two days later, cells were serum starved and left untreated (control) or were treated with phorbol 12-myristate 13-acetate (TPA) ( $1 \mu\text{g ml}^{-1}$ ) (TPA-pretreated). After 18 h, cells were untreated or exposed to carbachol ( $100 \mu\text{M}$ ), EGF ( $100 \text{ ng ml}^{-1}$ ), or TPA ( $50 \text{ ng ml}^{-1}$ ) as indicated for 5 min, lysed and ERK activity assayed on anti-HA immunoprecipitates as described in Fig. 1. Protein kinase C downregulation

by the 18-h treatment with TPA was confirmed by western blot analysis (data not shown). Data represent average  $\pm$ s.e.m. of triplicate samples. Similar results were obtained in 3 independent experiments. b, Effect of *ras*-inhibitory proteins on agonist-induced activation of ERK2. pcDNA (vector), m1, m2,  $\beta_1 + \gamma_2$  or activated *raf*(BXB-*raf*, ref. 30) containing plasmids ( $5 \mu\text{g per plate}$ ) were cotransfected with an expression vector carrying a dominant negative mutant of Ras (N17-*ras*)<sup>25</sup>, or rap-1a (ref. 26),  $5 \mu\text{g per plate}$ , as indicated. Cells were untreated or exposed to



stimulate biochemical pathways leading to ERK activation. Membrane association appears to be a requisite for this function.

We investigated whether protein kinase C (PKC) mediates ERK activation by G proteins in cells depleted of PKC by prolonged treatment with a high concentration of phorbol ester<sup>23</sup>. As shown in Fig. 4a, this procedure abolished ERK2 activation in response to a subsequent challenge with phorbol ester. In contrast, PKC downregulation only partially diminished activation of ERK2 mediated by m1 receptors, a finding consistent with previous reports<sup>6,24</sup>. Furthermore, we observed that PKC downregulation had little effect on ERK2 activation elicited by m2 or EGF receptors, or by overexpressed  $\beta\gamma$  subunits. Taken together these data demonstrate a PKC-independent pathway mediating signalling from G proteins to ERKs.

To explore the role of *ras* in ERK2-activation by heterotrimeric G proteins, we cotransfected COS-7 cells with m1, m2, or  $\beta\gamma$  cDNAs together with plasmids expressing *ras*-inhibitory proteins such as a dominant negative mutant of Ras (N17-*ras*)<sup>25</sup> and rap-1a (ref. 26). As shown in Fig. 4b, cotransfection of each *ras*-inhibiting construct nearly abolished ERK2 activation when induced through EGF, m1 and m2 receptors, or by  $\beta\gamma$  dimer overexpression, but had little effect on stimulation of ERK2 by activated *raf*. These findings provide strong evidence that *ras* function is required for transducing signals from G proteins to ERK2.

On the basis of our present study, activation of ERKs can be added to the growing list of effector functions mediated by  $\beta\gamma$ -heterodimers<sup>18</sup>. Furthermore, our findings also provide strong evidence that at least in COS-7 cells, free  $\beta\gamma$  dimers rather than activated  $\alpha$  subunits transfer signals from G-protein-linked receptors to ERKs.  $\beta\gamma$  subunits have been previously shown to mediate pheromone receptor activation of ERKs in the budding yeast *Saccharomyces cerevisiae*, although in this case the pathway is *ras*-independent<sup>27</sup>. In mammalian cells, however, signalling from  $\beta\gamma$  complexes to ERKs appears to require Ras, thus raising a mechanistic question regarding communication between  $\beta\gamma$  subunits and *ras*. In this regard, a recent finding has shown that  $\beta$  adrenergic-receptor kinase binds free  $\beta\gamma$  heterodimers through a region that contains a pleckstrin homology (PH) domain<sup>28</sup>. The majority of proteins known to regulate *ras*

carbachol ( $100 \mu\text{M}$ ) or EGF ( $100 \text{ ng ml}^{-1}$ ) for 5 min, lysed and ERK activity assayed on anti-HA immunoprecipitates as described in Fig. 1. Data represent the mean  $\pm$ s.e. of 3–5 independent experiments, expressed as a percentage of radioactivity incorporated into MBP as compared to cells transfected with vector DNA (100%). These same *ras*-inhibiting DNAs did not affect expression of HA-ERK2, m1 or m2 receptors, nor did they inhibit coupling to PI-PLC (data not shown).

activity also contain a PH domain<sup>29</sup>. Thus, we propose that free  $\beta\gamma$  subunits might affect functioning of Ras by binding the PH element of one or more *ras*-regulatory proteins. Such a hypothesis, linking G-protein-coupled receptors to small GTP-binding proteins, is testable and will be investigated in the future. □

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- Ray, L. B. & Sturgill, T. W. *Proc. natn. Acad. Sci. U.S.A.* **84**, 1502–1506 (1987).
- Cobb, M. H., Boulton, T. G. & Robbins, D. T. *Cell Regul.* **2**, 965–978 (1991).
- Pelech, S. L. *Curr. Biol.* **3**, 513–515 (1993).
- Davis, R. J. *J. biol. Chem.* **268**, 14553–14556 (1993).
- Schlessinger, J. *Trends biochem. Sci.* **18**, 273–275 (1993).
- Qian, N.-X., Winitz, S. & Johnson, G. L. *Proc. natn. Acad. Sci. U.S.A.* **90**, 4077–4081 (1993).
- Lange-Carter, C. A., Pleiman, C. M., Gardener, A. M., Blumer, K. J. & Johnson, G. L. *Science* **260**, 315–319 (1993).
- Cook, S. J., Rubinfeld, B., Albert, I. & McCormick, F. *EMBO J.* **12**, 3475–3485 (1993).
- Peralta, E. et al. *EMBO J.* **6**, 3923–3929 (1987).
- Her, J. H. et al. *Biochem. J.* **296**, 25–31 (1993).
- Uli, M. & Katada, T. *Adv. Second Messenger Phosphoprot. Res.* **24**, 63–69 (1990).
- Gilman, A. G. *Rev. Biochem.* **56**, 615–649 (1987).
- Bourne, H. R., Sanders, D. A. & McCormick, F. *Nature* **349**, 117–127 (1991).
- Hermouet, S., Merendino, J. J. Jr, Gutkind, J. S. & Spiegel, A. M. *Proc. natn. Acad. Sci. U.S.A.* **88**, 10455–10459 (1991).
- Kalinek, G., Nazari, A. J., Hermouet, S., Xu, N. & Gutkind, J. S. *Molec. cell. Biol.* **12**, 4687–4693 (1992).
- Strathmann, M. P. & Simon, M. I. *Proc. natn. Acad. Sci. U.S.A.* **88**, 5582–5586 (1991).
- Barbacid, M. A. *Rev. Biochem.* **56**, 779–827 (1987).
- Clapham, D. E. & Neer, E. J. *Nature* **365**, 403–406 (1993).
- Federman, A. D., Conklin, B. R., Schrader, K. A., Reed, R. R. & Bourne, H. R. *Nature* **356**, 159–161 (1992).
- Simonds, W. F., Butyrnski, J. E., Gautam, N., Unson, C. G. & Spiegel, A. M. *J. biol. Chem.* **266**, 5363–5366 (1991).
- Iniguez-Lluhi, J. A., Simon, M. I., Robinshaw, J. D. & Gilman, A. G. *J. biol. Chem.* **267**, 23409–23417 (1992).
- Pronin, A. N. & Gautam, N. *FEBS Lett.* **328**, 89–93 (1993).
- Coughlin, S. R., Lee, W. M. F., Williams, P. W., Giels, G. M. & Williams, L. T. *Cell* **43**, 243–251 (1985).
- Kahan, C., Seuwen, K., Melocke, S. & Pouyssegur, J. *J. biol. Chem.* **267**, 13369–13375 (1992).
- Feig, L. A. & Cooper, G. M. *Molec. cell. Biol.* **8**, 3235–3243 (1988).
- Kitayama, H., Sugimoto, Y., Matsuzaki, T., Ikawa, Y. & Noda, M. *Cell* **56**, 77–84 (1989).
- Errede, B. & Levin, D. E. *Curr. Opin. Cell Biol.* **5**, 254–260 (1993).
- Lefkowitz, R. J. *Cell* **74**, 409–412 (1993).
- Mussacchio, A., Gibson, T., Rice, P., Thompson, J. & Saraste, M. *Trends biochem. Sci.* **18**, 343–348 (1993).
- Bruder, J. T., Heidecker, G. & Rapp, U. R. *Genes Dev.* **6**, 545–556 (1992).

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# Rapid arrayed filter production using the 'ORCA' robot

Alex Copeland and Greg Lennon

**By adapting a commercially available, general purpose laboratory robot, it is possible to produce high-density gridded hybridization filters of clone colonies or DNA products. We are using this system to produce 60–90,  $8 \times 12$  cm filters in an 8-hour day, each containing 3,456 clones arranged in 96,  $6 \times 6$  grids.**

THE initial phases of our effort to map chromosome 19 at Lawrence Livermore National Laboratory (LLNL) involved constructing a cosmid-level map of the chromosome by fluorescent fingerprinting clones from a number of different flow-sorted chromosome 19 cosmid libraries and assembling the fingerprinted cosmids into contigs<sup>1</sup>. As part of our strategy for completing the physical map of chromosome 19, we are making extensive use of filter hybridization. Hybridization to high-density colony filters is used to verify contig assembly, to close gaps in the cosmid-level contig map, to identify contigs and cosmids containing genes of interest and to identify cDNAs. In this article, we describe increases in throughput made possible by adaptation of the ORCA robot (Hewlett-Packard, Palo Alto, California) to the task of producing high-density colony and DNA filters.

## Hardware and software

Chromosome-19 specific cosmid libraries have been described<sup>2</sup>. Cosmids are in the vector Lawrist 5 (ref. 3) or Lawrist 16 and are propagated in DH5 $\alpha$ F' bacteria (gift of Joel Jesse, GIBCO, Gaithersburg, Maryland). The combined libraries (29,664 clones) represent an approximately 12 $\times$  coverage of chromosome 19. Large format cDNA colony filters<sup>4</sup> are prepared from a normalized human infant brain cDNA library, kindly provided by M. B. Soares (Columbia University) and arrayed at LLNL. The library was constructed in Lafmid BA, and is propagated in DH10B (GIBCO). The current array contains 39,168 clones and forms the basis for the Integrated Molecular Analysis of Gene Expression (IMAGE) working group, which at the moment consists of 27 laboratories sharing sequence and map information. For all libraries, a dedicated replica set was used for plating.

Source plates are Falcon 96-well microtitre dishes (Becton Dickinson, Lincoln Park, New Jersey). The nylon Hybond-N membranes (Amersham, Arlington Heights, Illinois) supported on LB/agar (LB, 1% agarose, 50  $\mu$ g ml<sup>-1</sup> kanamycin or 100  $\mu$ g ml<sup>-1</sup>

ampicillin) are held in  $8 \times 12$  cm microtitre plate lids (USA Scientific, Ocala, Florida), for standard size filters, or  $17 \times 24$  cm Lexan dishes produced in house, for large format cDNA colony filters. Dishes are secured to the bench with adjustable braces. One to 18 microplates of the arrayed library are placed on the work surface between the sterilization station and the target dishes.

The ORCA system is composed of an optical bench, a 1- or 2-m rail, and a six-jointed arm (Fig. 1). The arm is capable of linear movement along the rail and rotation at its shoulder, elbow and hand joints. The wrist can rotate about the axis of the arm and can execute up to 77 rotations in either direction. The hand ends in a set of opposable fingers to which a tool may be attached

an interpreted language with approximately 90 per cent of the feature set capability of Microsoft QuickBasic<sup>5</sup>.

The pin tool is a Beckman high-density replication tool (HDRT, Beckman Instruments, Fullerton, California) which has 96, 0.016 inch stainless steel pins arranged in microtitre-plate format. The tool is attached to the ORCA with a simple adapter made at LLNL. The adapter has two tapped holes in the bottom for attachment to the pin plate holder and milled surfaces on either side which 'mate' with the finger blanks.

## Operation

To transfer colonies, the tool is sterilized and dried, dipped into the first source plate containing a control plasmid, then brought into position to touch lightly the surface of the nylon membrane of the first target filter. The tool is dipped again into the first plate and the first target filter is inoculated at a second position providing an internal control. The process is repeated for all the target filters, after which the tool is sterilized. Subsequently, the tool is dipped into each source plate and all target filters are inoculated at positions offset from the previous spots by 1.4 mm (Fig. 2a). The pin tool is sterilized by successive 5-s immersion in baths containing 1% bleach, water and 95% ethanol. After sterilization, the tool is dried for 25 s by a small fan. The water bath is flushed continuously to minimize the build-up of bleach.

Filter trays for cosmid plating are prepared as described previously<sup>2</sup>. Up to 45 filter trays are placed on the work surface. After all the source plates are transferred to the filters, the filter trays are inverted and incubated overnight at 37 °C. Colonies are fixed as previously described<sup>2</sup> except that the proteinase-K incubation was omitted. cDNA library colonies are plated on large format filters in  $17 \times 24 \times 1$  cm Lexan dishes made at LLNL. Eleven of these large filters can be made at one time. Each filter contains the equivalent of four of the smaller size filters. For both filter formats, duplicate spotting aids the scoring of hybridization positives (Fig. 2b).

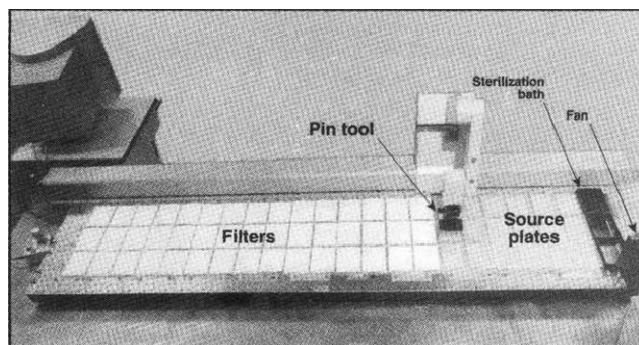


FIG. 1 The ORCA robot with the 2-m rail is shown configured for producing 45,  $8 \times 12$  cm filters. The sterilization bath can be seen on the far right-hand side of the photo; the fan is in the lower right-hand corner. The next six columns are the eighteen source plates to be transferred in duplicate. The target filters on agar occupy the remainder of the bench.

through interchangeable finger blanks. The arm receives instructions from an IBM PC or compatible computer running MS-DOS and Microsoft Windows 3.1. Robot motion commands are translated into motion profiles by a kinematics control unit connected to the host computer. The robot may be controlled directly from the command line or with a 'teach pendant', a joystick motion teaching tool.

The software was written with Hewlett-Packard's methods development software (MDS 2.0). MDS is a Windows 3.1-based development environment which includes a text editor, file management tools, tools for creating and modifying motions, and



FIG. 2a, Schematic of the layout of library plates on a filter spotted with a 6 × 6 grid. The first three and last three rows are duplicates, and the first off-set position is occupied by a control plasmid. A true positive will produce a signal in two locations. b, Autoradiograph of a typical hybridization using a random-primed cDNA probe (Prime-It, Stratagene, La Jolla, California). The film is overexposed to make the spot spacing apparent. Note that the two positive signals are in duplicate.

**METHODS.** Filters were prehybridized 6 h to overnight at 65 °C in 4XSSC, 1% SDS, 0.1 mg ml<sup>-1</sup> sheared denatured herring sperm DNA in

glass tubes in a rotisserie oven (Robbins Scientific, Sunnyvale, California). After pre-hybridization, denatured probe was added and the filters were hybridized overnight at 65 °C. Probes expected to contain repetitive elements were first preannealed in 4XSSC for 30–60 min with 0.5–1.0 mg ml<sup>-1</sup> Cot1 human DNA (GIBCO/BRL Life Technologies, Gaithersburg, Maryland) at 65 °C. Up to 20 filters were hybridized in the same 38 × 150 mm tube. Filters were washed briefly in 2× SSC at room temperature, 30–60 min in 2×SSC, 1% SDS at 65 °C in a Disc-wisk (Schleicher-Schuell, Keene, New Hampshire), and 15–20 min in 0.1×SSC, 1% SDS at 65–75 °C. A small amount, 1/20–1/50th of the total counts, of random-primed control plasmid was generally added to the hybridization to provide a reference grid. Positives were visualized by standard autoradiography using Kodak X-OMAT AR film (Eastman Kodak, Rochester, New York) or storage phosphor screen autoradiography on a PhosphorImager.

No special precautions are taken to prevent airborne contamination of the source plates and they remain open to the air during the plating procedure. To verify that no contamination of the source plates was occurring, eighteen plates containing LB without antibiotics were left out on the work surface for 4 h while the robot executed the spotting routine without dipping into the plates. The plates were then incubated overnight at 37 °C and inspected for growth. This experiment has been repeated several times, and thus far no growth has been observed (data not shown).

## Comparison

With a 96-pin tool it is possible manually to spot 5,000 clones per hour at a density of 1,920 clones per 8 × 12 cm filter<sup>6</sup>. Using our previously automated high-density colony filter production system, it is possible to produce up to 40 filters in an 8-h day, containing 3,456 clones per filter, using two robots and manually exchanging source plates every 1.5 min. At this level of production, continuous user attention is required. Unattended operation using a side loader (Beckman Instruments) is half as fast<sup>2</sup>, but makes possible unattended and overnight operation. The system described in this article allows production of up to 90 filters containing 3,456 clones per

filter in 8 h of unattended operation.

Our use of two different size filters streamlines the hybridization process. The large number of plates comprising the cDNA library, and the fact that most of our local users of filters made from this library rely on the PhosphorImager (Molecular Dynamics, Sunnyvale, California) for analysing the results, prompted us to adopt a format for the filters that minimizes the number of filters required for a whole library hybridization and makes most efficient use of the available PhosphorImager cassettes.

## Benefits

In addition to the increase in throughput and savings in user time, additional benefits made possible by use of this system include reducing the time that source plates remain at room temperature compared to previous methods, and maximizing the number of filters produced from each replica set per thawing. Our previous method required leaving thawed library plates at room temperature for 15 h or more to produce 30 filters at this density. We have found that library plates can be frozen and thawed up to three times before spotting quality declines. With the present system, 270 filters can be produced per set of library plates. We have recently begun colony spotting of

bacterial artificial chromosomes<sup>7</sup>, P1-derived artificial chromosomes<sup>8</sup>, and direct spotting of yeast artificial chromosome Alu-PCR products.

We expect that adapting the system so that it can accommodate 384-well plates will be quite simple. The necessary modifications to a 384-pin tool (General Atomic, San Diego, California) have already been made. Software modifications required are trivial. In order to maintain spacing between array units, slightly higher accuracy will be required compared to our present application. We plan on using the 384-well format as soon as higher volume square-well plates are commercially available, expected to be later this month. □

Alex Copeland and Greg Lennon are at the Human Genome Center, Biology and Biotechnology Research Program, Lawrence Livermore National Laboratory, L-452, Livermore, California 94550, USA. This work was performed under the auspices of the US Department of Energy by LLNL. We thank C. Prange for providing hybridization data. For more information, fill in reader service number 100.

1. Carrano, A. V. *et al.* *Genomics* **4**, 129–136 (1989).
2. Olsen, A. S. *et al.* *BioTechniques* **14**, 116–122 (1993).
3. Carrano, A. V. *et al.* *Genome* **31**, 1059–1065 (1989).
4. Lennon, G. G. & Lehrach, H. *TIGS* **7**, 314–317 (1991).
5. Schoeny, D. E. & Rollheiser, J. J. *Am. Lab.* **42–47** (1991).
6. Lennon, G. in *High Density Grid Technologies, Automated DNA Sequencing and Analysis* (ed. Adams, M.) 126–130 (Academic, London, 1994).
7. Shizuka, H. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **89**, 8794–8797 (1992).
8. Ioannou, P. *et al.* *Nature Genet.* **6**, 84–89 (1994).

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Reader Service No.4

# Advances on the analytical front

**Highlighted this week — a labstation that automates the upstream processes of DNA sequencing, a device for culturing hybridoma cells and an instrument for high-volume screening by fluorescence polarization in 96-well plates.**

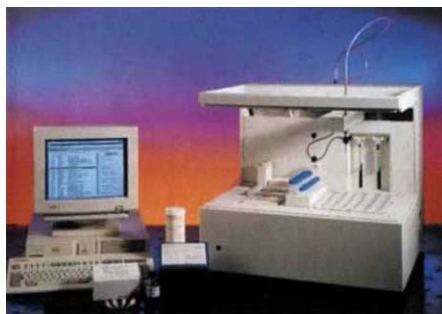
DESIGNED specifically for chemistry-related professionals, researchers, students and teachers, the model CH109 **chemical calculator** provides a scientific calculator and chemistry database — all in one instrument (*Reader Service No. 101*). Access to



**Portable calculator for solving chemical conundrums.**

empirical formulae and molecular weights is provided. In addition, the calculator provides elemental composition, conversion factors and fundamental constants, solvent boiling points and densities, and other chemistry-related computations. It incorporates an internal database of all 109 chemical elements, in addition to isotopes, amino acids, solvents, groups and ligands. Cost US\$280.

A new instrument launched jointly by Amersham and Molecular Dynamics is designed for **automating the upstream processes of DNA sequencing** (*Reader Service No. 102*). The Vistra DNA labstation, by automating mini-preps and sequencing reactions, is said to provide 'walk-away automation' up to the point of loading a gel.



**Amersham's Vistra DNA labstation.**

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Amersham says that up to 72 samples a day can be processed. Suggested initial applications include genome mapping and DNA-based diagnostic procedures. Incorporated in the chemistry of the labstation is Amersham's separation method, based on magnetic purification, which allows the isolation of single- or double-stranded DNA from phage or bacteria, without the need for centrifugation or extraction using organic solvents. For Sanger sequencing, the DNA sequencing enzyme Sequenase is used to label the DNA and prepare it for sequencing. This method is said to provide a long read-length (up to 500 bases). Alternatively, if the initial amount of DNA is low, or there is a high degree of secondary structure, the  $\Delta$ Taq cycle sequencing methodology can be adopted. Users are also offered a choice of using either radioactively- or fluorescently-labelled primers.

## ■ Immunology

Heraeus Instruments has introduced a new device for **culturing hybridoma cells** for the batch production of monoclonal antibodies (*Reader Service No. 103*). The miniPERM bioreactor is said to be capable of producing cell densities greater than  $10^7$



**Hybridoma cell culture system.**

cells per millilitre, and is designed to replace six standard roller bottles or several mice. A stated purity of output of 85 to 95 per cent should eliminate the need for purification procedures before screening. Multiple miniPERM units can be installed in a gassed incubator with a mini-PERM bottle roller.

Chemicon's **monoclonal antibody to slow myosin heavy chain** identifies Type-1 fibres (*Reader Service No. 104*). The monoclonal is said to react with both frozen and formalin-fixed material, making it suitable for use on archived samples. Chemicon says that it reacts with slow myosin heavy chain from a number of different species including human, rat and feline.

Anti E Tag antibody from Pharmacia Biotech is designed to support the company's recombinant phage antibody system in the capacity of **purification and detection of soluble single-chain antibody fragments** (ScFv) (*Reader Service No. 105*). The anti-



**Anti-E Tag antibody.**

body detects an 'E tag' present on ScFv expressed using the phagemid pCANTAB 5 E. It is intended for the detection of ScFv in applications such as western blotting and ELISAs. Anti E Tag antibody can also be attached to a matrix for affinity purification of ScFv produced using the system.

Ancell's new research **products for human T-cell/B-cell interaction** include monoclonal antibodies to CD80 and anti-CD40 (*Reader Service No. 106*). Other monoclonal antibodies available include CD24, T-cell receptor, selected integrins, selected adhesion molecules, B- and T-cell markers. Soluble human CD4 and soluble human CD54 (ICAM-1) will be the first in a line of soluble human CD molecules available for research purposes.

## ■ Laboratory hardware

If you are working with pre-coated magnetic beads, then the new Skatron SkanWasher, a dedicated **automatic washer for magnetic beads in microplates**, may be of interest



**Skatron's SkanWasher.**



(Reader Service No. 107). Coated magnetic beads are mixed and incubated with sample in a 96-well microplate. Magnets trap the beads with bound material in a pellet, which can be moved along the bottom of the well. Aspiration of surplus liquid and unbound material is done automatically, and without disturbing the pellet, says Skatron. Wash buffer is then added for further purification. The pellet can be resuspended and the washing steps repeated. Other design features include a removable wash head, adjustable vacuum and pressure, adjustable position of the microplate and a motorized plate lift that operates during wash cycles.

Two brochures from BioLogic describe the latest products in the company's electrophysiology line: the RK-400 **patch-clamp amplifier** and the RSC-100 **rapid solution changer** (Reader Service No. 108). The RK-400 offers a capacitive feedback mode for small current, single-channel recordings and a resistive feedback mode for the study of higher conductance channels or for whole-cell clamp. Other design features include 50 kHz bandwidth in all modes, measurements of cell capacitance and series resistance, boosted series resistance compensation in whole-cell mode independent from its measurement, independent holding voltage and holding current controls, a remote-controlled V-clamp/I-clamp switch, a 'zap' facility for easier cell penetration, and a dual-scale internal pulse generator. The RSC-100 system is designed for rapid, multiple and programmable solution exchange at the patch-clamp pipette tip. Excised-patch mode and whole-cell mode are both accommodated. Windows 3.1-based software permits computer control of the instrument.

Jolley Consulting and Research's Fluorolite FPM-2 permits high-volume **screening by fluorescence polarization** in 96-well microtitre plates (Reader Service No. 109). Stated applications include studies involving drug discovery, DNA and protein binding, proteases, receptor/ligand binding, antibody/antigen interactions and screening procedures, immunology and toxicology investigations. With fluorescence polarization, the company says that no washing or intermediate steps are required: the fluorescent-labelled ligand is added to its unlabelled receptor, and an increase in fluorescence polarization indicates binding. This reaction



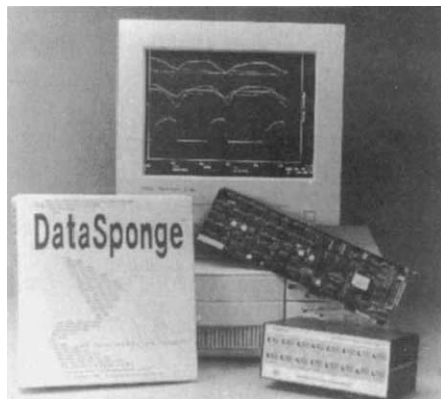
**Fluorolite FPM-2 screening system using fluorescence polarization.**

may be inhibited by unlabelled ligand in a competitive format.

The new LaserScissors **laser microdissection system** from Cell Robotics can be used as a standalone research tool or in conjunction with the company's companion products, the LaserTweezers 1000 and 2000 optical trapping systems (Reader Service No. 110). The company says that the new device will allow researchers to make sub-micron incisions in cells, cell processes (such as neurites) or organelles (for example, chromosome microdissections), and will also ablate unwanted cells from among heterogeneous cultures. The LaserScissors module, including its focusing and steering optics, mounts directly on a standard microscope. By using a pulsed nitrogen dye laser with a standard emission wavelength of 390 nm, the device can make 3–4-nanosecond incisions (spot size of less than 0.5 microns), says CRI. Emission wavelengths ranging from near-ultraviolet to visible can be varied using interchangeable dye cuvettes. This allows the emission wavelength to be selected on the basis of application.

## Software

DataSponge is a **data acquisition software package** for IBM or compatible computers that is intended for high-volume, high-speed applications in the life sciences (Reader Service No. 111). In addition to increased speed and full Postscript support, the soft-

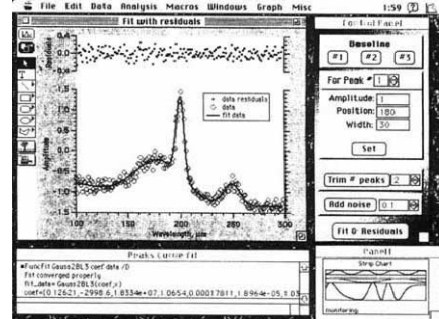


**DataSponge data acquisition package for IBM and compatible personal computers.**

ware is also said to feature a quicker user interface, 'on-the-fly' annotation of data, a high-resolution colour display, an optional x-y oscilloscope display, digital readouts during sampling or playback using smart cursors and mouse, context-sensitive help, and networking capabilities. Features of this research tool that have been retained include easy A/D board setup and preparation for sampling and real-time graphic calibration. The software provides simultaneous storage to hard disk and lag-free display — even while sampling at the A/D board's maximum rate, says WPI. Also featured are real-time VCR-style screen review of acquired data, the ability to transfer data to spreadsheet

or analysis programs, and a variety of laser-printing options.

Four years in the making, WaveMetrics is now shipping Igor Pro 2.0 — the company's major upgrade to its programmable **scientific graphing and data analysis application** for the Macintosh (Reader Service No. 112). In order to satisfy the demands of scientific journals, Igor Pro 2.0

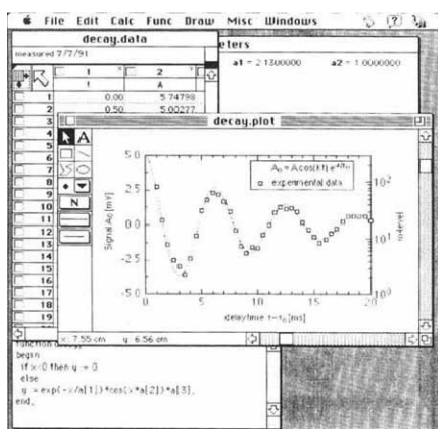


**Igor Pro 2.0 now shipping.**

is said to concentrate on providing extensive control of x,y graphs. Graphics can be exported as EPS files. Features of this latest version, which supports System 7, include multiple positionable axes, additional markers, text rotation, interactive and programmable drawing tools with multiple coordinate systems, user-defined buttons and readouts, and support for data acquisition. Igor Pro costs US\$495; upgrades are available for US\$200. A Power-Mac native-mode Igor Pro is planned for release later this year.

Ultimage is Graftek's **image processing and analysis package** for Apple Computer's newest Macintosh computers based on PowerPC technology (Reader Service No. 113). The software has an assortment of tools and includes a library of over 400 image acquisition, enhancement and display functions which handle 1-, 8-, 16-, 24- and 32-bit images in a variety of file formats. It is also said to be well-suited for a variety of particle detection, measurement and analysis tasks: algorithms can handle more than 60 shape and density measurements for each object recognized in an image. The software also includes many common imaging functions such as convolution and nonlinear filters, geometrical transforms, thresholding and colour-space conversions. In addition, Ultimage can be used to produce statistical reports and charts, and can export image processing results to spreadsheets or other Macintosh applications for further analysis.

QuantumSoft is now shipping version 4.1 of ProFit, a **software package for scientific data analysis** for the Macintosh (Reader Service No. 114). The program's main features are a drawing editor for creating presentations of data and functions of a quality suitable for publication; a mathematical kernel for curve fitting, functional analysis, data transformation and statistical



ProFit curve-fitting software for the Mac.

operations; a built-in compiler for defining arbitrary mathematical functions and transformation algorithms in a subset of the Pascal programming language; and linking of external, user-defined code modules. ProFit allows the exchange of data and pictures with most Macintosh applications.

TINA is Raytest's new PC-based software for one- and two-dimensional thin-layer chromatography applications (Reader Service No. 115). The package is intended for use with Fuji's desktop Bio-Imaging analyser, the BAS 1000, which uses a reusable phosphor imaging plate for sensing radioactive energy.

D-2000 is an artificial neural systems- (ANS) based product from Design Sciences for modelling complex processes and building data-driven support systems for scientific research (Reader Service No. 116). It allows researchers to experiment with pattern recognition-based approaches to solving problems in molecular modelling, simulation of biological systems, analysis of genetic sequences and image analysis. D-2000 is supplied with five ANS models available for the analysis of large and/or complex data sets and an icon-oriented graphical user interface. It works with data from sources such as databases, spreadsheets, image files, expert system shells and simulation/modelling tools. A 386/486 PC and OS/2 or Windows 3.1 are required.

## Image analysis

Synoptics has added a new high-resolution, three-chip colour camera to the range of facilities for VIDS, its semi-automatic image analysis system designed for applications in histology and cytology (Reader Service No. 117). VIDS, compatible with computers with a PC and MCA architecture, consists of an interface card, a video monitor, digitizing pad and cursor, as well as the camera. The digitizing pad and cursor is designed to be used interactively by the user to define the features of interest for subsequent measurement and analysis by VIDS software. Measurement capabilities include area, perimeter, width, roundness, angle and

equivalent spherical volume. Results are continuously displayed on the monitor and automatically stored. Included among the data analysis and display facilities are one- and two-way analysis of variance, *t*-test, frequency distribution histograms, correlation coefficients, scatter diagrams and particle size distributions. Data can be exported to a spreadsheet program such as Excel.

The MiraCal system from Life Sciences Resources is an acquisition and analysis package for spatial and temporal ratio-metric imaging of the new generation of optical probes for use on living cells (Reader Service No. 118). The system includes a cooled, low-noise, 8-bit fast readout imaging camera for living cell work, which is said to be capable of full imaging and 'sub-array' scanning. Typical applications include imaging and calibrated measurement of ion-sensitive optical probes such as Fura-2 and BCECF, and imaging fluorescent markers such as fluorescein and rhodamine. The system, which mounts on most conventional upright or inverted-stage microscopes, supports single or multiple probes using up to four wavelengths of excitation, and both dual and single emission probes.

## Physical sciences

Coherent Laser Group has two new additions to its laser product line: a pulsed Nd:YAG laser and a solid-state, continuous-wave blue laser (Reader Service No. 119). The Infinity 40-100 pulsed Nd:YAG laser utilizes a phase conjugate mirror and a

relay imaging system to achieve 40 W infrared output at 100 Hz with a near diffraction-limited beam. The company's D<sup>3</sup> directly doubled diode laser is designed to produce greater than 10 mW of continuous-wave output, at 430 nm. The D<sup>3</sup> technology is also said to provide a significant reduction in power consumption (20–50 times less than that of an air-cooled ion laser).

For the vacuum deposition community, CVT is offering new high-temperature effusion sources (Reader Service No. 120). The KC2010 10-cc capacity high-temperature effusion cell can be retrofitted to all vacuum systems equipped with standard NW35 ports (70 mm outer diameter). CVT says stability of the element allows operational temperatures up to 2,000 °C; elimination of insulators from the 'hot' zone reduces outgassing, providing minimal contamination; and a high number of radiation shields reduce the amount of heat loss to the system (typically 800 W to achieve 2000 °C). The cylindrical element is said to form a near-perfect black body radiator; this provides efficient thermal coupling to the crucible allowing the element to run at much lower temperatures than conventional wire-wound sources. □

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# Watershed for the structure of cytochrome *c*

**Redox-dependent changes in the position of bound water inside cytochrome *c* in solution may be important for its function as an electron transfer protein.**

THE importance of water to the structure and function of biological molecules is well established; however, the view of water as a lumpy continuum surrounding, or surrounded by, biological molecules is not only inaccurate but may also hinder our comprehension of the inner workings of the molecular machinery. Now, thanks to increasingly sophisticated techniques for structure determination, the nature of water's contribution is becoming clearer. In a paper in this month's *Nature Structural Biology*<sup>1</sup>, Qi *et al.* exploit the ability of NMR to measure specific classes of atomic interaction in order to observe the position of structurally important water molecules in the haem-containing electron transport protein cytochrome *c*. What is more, they identify changes in the position of some water molecules in the oxidized and reduced forms of the protein, which may well have a bearing on its function.

The ability to detect and position water molecules within protein structures is not new. Electron density maps derived from X-ray diffraction of protein crystals invariably show a layer of highly ordered water molecules coating the surface of the protein and filling any available holes or crevices (reviewed in ref. 2). Owing to the nature of the crystallographic experiment, however, it is hard to assign biological significance to these water molecules, even when X-ray diffraction is complemented by neutron diffraction to reveal the positions of the hydrogen as well as the oxygen atoms of water. The structures derived from X-ray crystallography are averaged over times measured in hours, so the presence of well-defined water implies that there is a water molecule at that position for most of the time in most of the protein molecules, and not that the water is necessarily tightly held there. This can evoke an impression of water networks that are more highly ordered than the *in vivo* arrangement.

Also in this month's *Nature Structural Biology*: alteration of the substrate specificity of a serine proteinase; peptide helices in membrane environments; determination of the structure of two new zinc-finger domains; long-range effects of single amino-acid substitutions on the conformations of peptides; and an empirical algorithm for the determination of helical propensity.

Investigation of the structure of water in proteins by NMR has been hampered by problems in resolving signals due to bound water molecules from those arising from bulk solvent. The development of multidimensional NMR techniques has allowed the detection of long-lived water in proteins such as pancreatic trypsin inhibitor<sup>3</sup>, interleukin-1 $\beta$ <sup>4</sup> and FK506-binding protein<sup>5</sup>. In general, the positioning of these water molecules is the same as in the X-ray crystallographic structures. Qi *et al.*<sup>1</sup> have used a combination of nuclear Overhauser enhancement (NOE) and total correlation spectroscopy, in what is essentially a filtered two-dimensional experiment, to identify six water molecules in ferrocyanochrome *c* and five in ferricytochrome *c* that have residence times longer than 300 ps.

Early X-ray crystallographic studies of cytochrome *c* from tuna<sup>6,7</sup> revealed four deeply buried water molecules, one of which was implicated in setting the redox potential of the haem group. But as unnaturally high salt concentrations were used to facilitate crystallization, the relevance to the situation *in vivo* was uncertain. Qi and colleagues also detected these four water molecules, but could not fully interpret their NMR spectra without including other long-lived water molecules (two in ferrocyanochrome *c* and one in ferricytochrome *c*).

Four of the water molecules in cytochrome *c* appear to be classic examples of structural water filling holes inside the protein. As such they are unlikely to fulfil a functional role, although they must be important to the stability and integrity of the protein. It is in fact one of these waters that escapes detection in the oxidized form of the protein: it sits in a loop region close to the surface of the protein, so this elusiveness may reflect a decreased residence time in ferricytochrome *c* rather than its absence from this site.

The other two water molecules lie in the haem-binding cleft of cytochrome *c* and are thus of greater functional interest. One of these had previously not been spotted by X-ray crystallography and could now help resolve a long-standing inconsistency between theory and experiment. Earlier estimates of the solvent reorganization energy based on models of cytochrome *c* with only one water molecule bound in the haem cleft were too low: the extra water should correct the discrepancy.

The second water molecule in the haem cleft, originally postulated to influence the redox potential, lies against the haem ring. It changes position according to the oxidation state of the protein: upon oxidation the water moves 3.7 Å further away from the haem iron (from 5.6 Å to 9.3 Å). This movement alters the entire hydrogen-bonding structure within the cleft. It is important that the NOE distance restraints defining the water's position involve quite different residues in the oxidized and reduced protein. Changes in the electronic structure so close to the haem iron will profoundly affect the redox potential of the haem.

The resurgence of interest in biological electron transfer is being fuelled by studies like this. With each new piece of data it is becoming increasingly clear that the former pre-eminence of X-ray crystallography in structure determination, focusing as it does on the semi-rigid regions of the peptide scaffold, may well have adversely constrained our thinking in this area.

Improvements in molecular dynamics simulations and the flourishing of NMR spectroscopy offer a more mobile picture of biological molecules. For example, the idea of a fixed complex between cytochrome *c* and cytochrome *c* oxidase, inferred from X-ray crystallography, has recently been called into question<sup>8</sup>. Consequently the popular concept of 'molecular wires', or predetermined covalent pathways that effect electron transfer between redox donors and acceptors in the respiratory chain, is now open to debate.

Cytochrome *c* has shown us that a protein's water can be used in more than a purely structural role. Evidently we must consider the structure and dynamics of the whole protein, non-peptide as well as peptide.

**Christopher Surridge**

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1. Qi, P.X., Urbauer, J.L., Fuentes, E.J., Leopold, M.F. & Wand A.J. *Nature struct. Biol.* **1**, 379–383 (1994).
2. Levitt, M. & Park, B.H. *Structure* **1**, 223–226 (1993).
3. Otting, G. & Wuthrich, K. *J. Am. chem. Soc.* **111**, 1871–1875 (1989).
4. Clore, G.M., Bax, A., Wingfield, P.T. & Gronenborn, A.M. *Biochemistry* **29** 5671–5676 (1990).
5. Xu, R.X., Meadows, R.P. & Fesik, S.W. *Biochemistry* **32**, 2473–2480 (1993).
6. Takano, T. & Dickerson, R.E. *J. molec. Biol.* **153**, 79–94 (1981).
7. Takano, T. & Dickerson, R.E. *J. molec. Biol.* **153**, 95–114 (1981).
8. Jeng, M-F., Englander, S.W., Pardue, K., Rogalsky, J.S. & McLendon, G. *Nature struct. Biol.* **1**, 234–238 (1994).